Molecular Analysis of Podocyte Genes in Nephrotic Syndromes

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Molecular Analysis of Podocyte Genes in Nephrotic Syndromes

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Contents

List of abbreviations

ACTN4	gene encoding alpha-actinin 4
AMRF	action myoclonus-renal failure
bp	base pairs
CD2AP	CD2 associated protein
CMS	Cas ligand with multiple SH3 domains
CNF	congenital nephrotic syndrome of the Finnish type
CoQ2	*para*-hydroxybenzoate-polyprenyl transferase
CoQ10	coenzyme Q10
DDS	Denys-Drash syndrome
DMS	diffuse mesangial sclerosis
DNA	deoxyribonucleic acid
ESRD	end stage renal disease
F-actin	filamentous actin
FS	Frasier syndrome
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
GFR	glomerular filtration rate
GMS	Galloway-Mowat syndrome
kD	kilodalton
LAMB2	gene encoding laminin β2 chain
LMX1B	LIM homeobox transcription factor 1 beta
MCNS	minimal change nephrotic syndrome
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
MYH9	gene encoding the heavy chain of nonmuscle myosinIIA
NPHS1	gene encoding nephrin
NPHS2	gene encoding podocin
NS	nephrotic syndrome
PAN	puromycin aminonucleoside
PLCE1	gene encoding phospholipase C epsilon
SCARB2	gene encoding lysosomal integral membrane protein type 2
SH3	Src homology 3
SRN1	steroid resistant nephrotic syndrome
tRNA	transfer ribonucleic acid
TRPC6	gene encoding transient receptor potential cation channel 6
WT1	gene encoding Wilms tumor 1

Chapter 1

Introduction and outline of the thesis

Kidneys play an important role in maintaining salt and water balance, and removing toxic waste products. The functional unit of a kidney is called the nephron. It is composed of different sections; the glomerulus, proximal tubule, loop of Henle, distal tubule and collecting duct, all contributing to the renal clearance. The glomerulus consists of a cluster of capillaries that branches from the afferent arteriole and is surrounded by the Bowman's capsule. Driven by a local high blood pressure, water and small molecules are filtered from the glomerulus and are collected in the Bowman's capsule to form primary urine. Then, passing the tubular sections, several secretion and (re)absorption processes determine the final composition of the urine.
This thesis focuses on the structure and function of the glomerular filter, and more in detail the epithelial cells called podocytes, and their role in developing the histological finding focal segmental glomerulosclerosis (FSGS).

The Glomerular Filtration Barrier

The glomerulus consists of a cluster of capillaries appearing in a looped formation supported by mesangial cells. While blood plasma passes the glomerular capillary loops, the local intracapillary pressure drives plasma through the glomerular filtration barrier which consists of at least three layers (figure 1). A recent review by Salmon *et al* describes new insights in the structure of the glomerular filtration barrier and distinguishes two additional layers which play an important role in permeability [1].

The glomerular endothelial cells, which separate the blood and tissue compartments, represent the traditional first layer. The endothelial cells are highly flattened cells and regulate vasomotor tone and haemostasis [2]. The role of the endothelial cells in selective filtration seems not to be substantial since they are highly fenestrated and highly permeable to water and small solutes. However, a recent study has shown that morphological alterations in the endothelial cell glycocalyx, making the cell surface extremely negatively charged, have functional consequences for glomerular permeability [3]. Using the GAG-degrading enzyme chondroitinase decreasing the thickness of the endothelial cell glycocalyx resulted in an increase of albumin clearance [3]. This endothelium surface layer, composed of the endothelial-cell glycocalyx, secreted proteoglycans, and trapped plasma constituents, now emerges as an additional glomerular filtration barrier [1]. The arrangement and density of these constituents influences the degree of molecular sieving [1].

The endothelium is completely surrounded by the glomerular basement membrane (GBM). This dense structure of extracellular matrix components provides structural support for the capillary wall necessary to maintain local high blood pressure. The main components of the GBM (collagen type IV, laminins, nidogen, and proteoglycans) contribute to the selective permeability of the GBM based on size and charge [4]. In the past years, many studies were focussing on the structure of the GBM because it was considered to be the leading part of the glomerular filtration barrier. Although structural abnormalities in the GBM may lead to proteinuria and hematuria, as it occurs in Alport's syndrome (mainly due to collagen IV α5 chain mutations, but also collagen IV α3 and α4 chains) or Pierson's syndrome (due to laminin β2 mutations) [5,6], the discovery of several novel proteins important for glomerular permeability made the podocytes the favourite candidate for constituting the main part of the glomerular filtration barrier.

The podocytes, or visceral epithelial cells, represent the next layer. They are highly specialized, terminally differentiated cells with cytoplasmic extensions, the so-called foot processes. Podocytes have an important role in size and charge selective permeability, but also in synthesising and maintaining the GBM [7]. Furthermore the fenestration of endothelial cells depends most notably on vascular endothelial growth factor A (VEGF-A) secreted from differentiated podocytes [2].

The foot processes of the podocytes attach to the outer surface of the GBM through cell membrane receptors (α3β1 integrins linked to talin, vinculin and paxillin, and α-, and β-dystroglycans linked to utrophin, see figure 2) [8]. Adjacent foot processes interdigitate, forming a pore of about 25-40 nm in width. This pore, or slit, is covered by a membrane with

a “zipper-like” structure [9]. When primary urine passes the podocyte slit membrane it enters the urinary space and it is generally assumed that no further significant resistance to fluid movement occurs until the exit into the proximal tubule. However, serial section transmission electron microscopy revealed three ultrastructurally distinct urinary spaces of which the subpodocyte space covers approximately 60% [1,10]. The subpodocyte space is defined by the space between the podocyte cell body and foot processes. Primary urine exits this space to the next urinary division through a narrow pore-like structure at the periphery of the podocyte by extravascular pressure regulated by this exit pore [1,10]. Further studies are required to determine the exact role of the subpodocyte space in the glomerular filtration barrier.

Recently, the structure of the slit membrane became the focus of many studies, and although the complete structure is not elucidated yet, several new proteins were found to be important for its function. Many components of the slit membrane are involved in the pathogenesis of (nephrotic range) proteinuria.

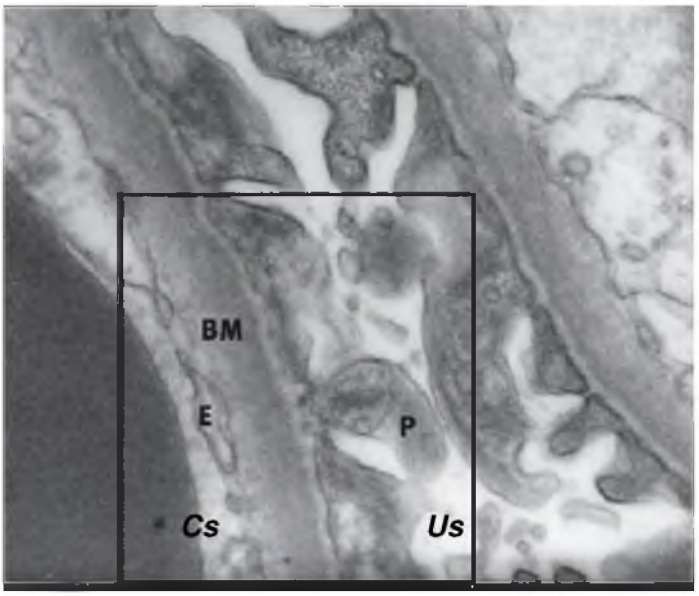

Figure 1 A cross section (electron microscopy, original magnification 30,000x) of the glomerular filtration barrier with the capillary space (*Cs*), urinary space (*Us*), endothelial cells (E), glomerular basement membrane (BM), and podocytes (P).

Nephrotic Syndrome - Focal Segmental Glomerulosclerosis

A nephrotic syndrome (NS) is defined by the occurrence of heavy proteinuria, oedema, and hypoalbuminaemia, and is classified as steroid-sensitive or steroid-resistant. In children NS occurs most commonly at a young age, with a peak incidence at two years. In most cases (~80%, according to the International Study of Kidney Disease in Children (1967-1974),

ISKDC) patients have minimal change NS (MCNS), which is characterized by responsiveness to steroid treatment accompanied with more or less frequent relapses. In 5-10% of NS patients (ISKDC) focal segmental glomerulosclerosis (FSGS) is found [11,12]. Less frequent histologic lesions are mesangial proliferative glomerulonephritis, membranoproliferative glomerulonephritis, and membranous glomerulopathy [11].

FSGS is a histological finding commonly seen in a large variety of conditions with different underlying causes. These conditions share the focal (only some of the glomeruli are involved) segmental (only part of an entire glomerulus is involved) sclerosis of the glomerular capillary tuft and manifests with proteinuria. As the disease progresses, the sclerosis has a more diffuse and global pattern. The biopsy may show mesangial deposits of immunoglobulin M or complement C3 and hyaline deposits all over the capillary loops [13]. Podocyte alterations (like foot process effacement) are most notably in FSGS.

Recently, FSGS has been classified in five sub-categories: collapsing variant, tip lesion variant, cellular variant, perihilar variant, and FSGS not otherwise specified (NOS) [14,15 for further reading]. Whether subdividing FSGS in different subcategories gives insight in clinical features and renal outcome is a subject of several reports recently reviewed by D'Agati [16]. Collected data from adult patients with biopsy proven FSGS showed a predisposition of younger and more often African-American patients for the collapsing variant of FSGS [17]. The collapsing and tip variants usually manifest with more severe proteinuria compared to the perihilar and NOS variants. Patients with the last two morphologic variants tend to have higher blood pressure and pathologically more arteriosclerosis [17]. As far as renal outcome concerns, the patients with tip lesion variant of FSGS seem more often to achieve complete remission, whereas the collapsing variant of FSGS has worse renal survival rates [17]. It is not excluded that different morphologic variants represent different stages of disease progression in FSGS.

FSGS commonly progresses to end stage renal disease (ESRD) requiring dialysis or renal transplantation. Post-transplant recurrence occurs in 35-40% of patients with idiopathic FSGS [18,19], mainly a few days after transplantation, and is often sensitive to plasmapheresis and cyclophosphamide treatment [20-22]. The recurrence may be due to a circulating plasma factor [20]. This factor may be responsible for an increase in podocyte integrin-linked kinase activity leading to podocyte detachment from the GBM as found in some patients [23]. Recently, cardiotrophin like cytokine-1has been identified as being a candidate for the FSGS permeability factor [24].

Lately, the role of Notch signaling, which is involved in the regulation of many cellular processes like proliferation, differentiation, and cell death [25], in the development of renal diseases is debated. Although NotchI protein plays a crucial role during kidney development, very little active NotchI can be detected in mature kidneys [26]. In glomerular epithelial cells of patients with diabetic nephropathy or FSGS, however, an increased presence of the active protein domain of NotchI was found, which, based on the homology with mouse podocyte biology [27], may be involved in apoptosis induction.

Another classification of FSGS is based on the underlying cause and subdivides it into three categories: idiopathic, genetic, and secondary due to injury, medication, or drug

abuse [13,16]. The last years genetic linkage studies have identified several genes involved in the development of FSGS and have contributed to the understanding of its pathophysiology (table 1). A subset of genes enlisted in table 1 are discussed below in more detail and figured in figure 2.

NPHS1

One of the major components of the slit membrane is the transmembrane protein nephrin (*NPHS1).* Nephrin has an important role in maintaining the structure of the podocyte slit membrane, as shown by nephrin deficient mice which develop proteinuria and foot process effacement [28]. Injection of anti-nephrin antibody in animals also results in foot process effacement [29]. Intracellularly, nephrin functions as a signalling molecule [30,31]. Nephrin (and also CD2AP, another component of the slit membrane) associates with the p85 regulatory subunit of phosphoinositide 3-OH kinase (PI3K) thereby stimulating the AKT signalling pathway controlling cell growth, migration, and survival [30].

Nephrin oligomers associate with lipid rafts in the slit membrane [32]. A rat model of antibody (recognizing a podocyte-specific 9-O-acetylated GD3 ganglioside) induced foot process effacement showed morphological changes of the filtration slits with apical dislocation and tyrosine phosphorylation of nephrin [32]. As such, tyrosine phosphorylation may regulate the subcellular redistribution of slit membrane proteins [32,33]. Furthermore, a study in Fyn deficient mice, showed an important role for tyrosine phosphorylation in nephrin-dependent intracellular signalling [34]. Fyn, a member of the Src protein kinase family, tyrosine phosphorylates the cytoplasmic domain of nephrin and Fyn deficient mice develop proteinuria and foot process effacement [34].

NPHS1 was found mutated in patients with the congenital nephrotic syndrome of the Finnish type (CNF). CNF has an incidence of 1:10,000 births in Finland, but less frequently in other countries. Although several missense mutations were found in CNF patients, two mutations, the Fin_{major} (deletion nucleotides 121 and 122) and Fin_{minor} (premature stop at amino acid 1109), account for over 90% of the cases in Finland [35]. Recurrence of CNF may occur in 20-25% of the patients after receiving a renal allograft and may be caused by anti-nephrin antibodies [36,37]. In addition to the renal disease, patients with *NPHS1* mutations may also show neurological symptoms including muscular hypotonia, dyskinesia, mild cerebral atrophy and hearing impairment of still obscure origin [38].

NPHS2

Podocin (*NPHS2*), a member of the stomatin protein family, is exclusively expressed in the podocytes and localizes at the insertion of the slit membrane. Due to its similarity to stomatin, it is believed that podocin forms a hairpin like structure with intracellular NH_2- and COOH-termini [39,40].

Podocin, like nephrin, associates with lipid rafts [39], and recruits nephrin and CD2AP in these rafts ensuring a stable and proper functioning filtration barrier. The COOH- terminal cytoplasmic tail of podocin interacts with nephrin and Cd2ap (the mouse homolog) [41]. This protein interaction greatly enhances nephrin-induced signalling *in vitro* [31]. The COOH-terminal domain of podocin also binds NEPH-1, a podocyte slit membrane protein structurally related to nephrin [42]. NEPH-1 is involved in maintaining the structure of the filtration barrier and also interacts with nephrin [43].

Podocin dysfunction leads to alterations of the slit membrane assembly and to proteinuria in experimental models. $NPHS2^{-/-}$ mice develop proteinuria and massive mesangial sclerosis (different from FSGS seen in humans), the podocytes are enlarged and focally vacuolized. The sclerosis rapidly progresses with age. Beside the absence of podocin, no nephrin is found in the foot processes as well. The podocin deficient mice die a few days after birth [44].

In human, *NPHS2* mutations, are mainly associated with autosomal recessive steroid-resistant nephrotic syndrome (SRN1) [38], but were also found in sporadic cases of steroid resistant nephrotic syndrome patients [45-48]. A recent study has demonstrated that also milder *NPHS1* mutations can cause a childhood-onset steroid-resistant nephrotic syndrome with underlying histological lesions ranging from minimal change nephropathy to FSGS [49].

The podocin variant R229Q is often found and has an allele frequency of 3.6% in a control population. The mutant protein has a decreased binding efficiency to nephrin and enhances FSGS susceptibility in association with a second *NPHS2* mutation [47]. The R229Q variant is also associated with microalbuminuria in the general population [50].

Mutated podocin may cause a disturbance in recruiting nephrin to the plasma membrane [51,52], which is comparable with the lack of nephrin in the slit membrane found in podocin deficient mice [44]. The R138Q NPHS2 mutant (one of the most common found mutation) results in retainment of the mutant podocin in the endoplasmatic reticulum. The R138X NPHS2 mutant yields a mutant podocin protein that is not able to associate with lipid rafts in the plasma membrane. Both mutant proteins were unable to recruit nephrin to the lipid rafts and lost their ability to enhance nephrin signaling [51,52]. NH_2-terminal mutant proteins were still able to associate with lipid rafts and had no effect on nephrin localization [53]. Collected clinical data from patients with *NPHS2* mutations showed that podocin mutants retained in the endoplasmatic reticulum are associated with earlier onset of the disease than those correctly targeted to the cell membrane [52].

Early reports showed that patients with *NPHS2* mutations had no recurrence of FSGS after renal transplantation. Now it is believed that patients with *NPHS2* mutations have a lower risk for recurrent FSGS after renal transplantation compared to patients with idiopathic

FSGS [54-56]. Patients carrying a heterozygous *NPHS2* mutation show a higher risk for recurrent FSGS (5 out of 8 published) in contrast to patients with homozygous or compound heterozygous mutations (5 out of 68 published) [57]. The authors state that grafts from carriers of *NPHS2* mutations, such as from parents, should be avoided because of the higher risk of recurrent FSGS. Furthermore, carriers of heterozygous *NPHS2* mutations should be strictly monitored in the post-graft phase [57].

CD2AP

Although first found in a yeast two-hybrid screen as a protein binding to the T-cell membrane protein CD2 during cell-cell interaction [58], an important role for Cd2ap (CD2 associated protein) in the kidney became evident when Cd2ap knockout mice died because of renal failure involving the glomerulus [59]. The $Cd2ap^{-/-}$ mice died at an age of 6 to 7 weeks, but already at one week glomeruli were increased in size and cellularity (mesangial cell proliferation and glomerulosclerosis) and electron microscopy showed extensive foot processes effacement [59]. $Cd2ap^{+/-}$ mice showed no proteinuria, but have an increased susceptibility to glomerular injury by immune complexes and nephrotoxic antibodies [60]. At 9 months of age, glomerular lesions were present with an increase in mesangial cellularity. Some lesions were similar to human FSGS and for this reason primary FSGS patients were tested for mutations in the *CD2AP* gene (the human homolog of Cd2ap, originally named CMS) [60] One heterozygous splice-site mutation was detected in two patients. The predicted mutated protein would lack more than 80% of the protein. Immunoblotting CD2AP isolated from immortalized B-lymphocytes showed a reduction in CD2AP expression in these patients [60].

CD2AP/Cd2ap is a multifunctional adaptor molecule localized to the cytoplasm, membrane ruffles, and leading edges of cells [61]. The protein plays a role in cytoskeletal remodeling [56,59], cell survival [30,62], and endocytosis [63-65]. The COOH-terminus of CD2AP directly interacts with the cytoskeletal protein filamentous actin (F-actin) [61,66] and synaptopodin, an actin bundling protein [67]. At the slit membrane, CD2AP interacts with nephrin and podocin [41,68] and serves as a linker anchoring slit membrane proteins to the actin cytoskeleton of podocytes.

CD2AP and nephrin bind to the p85 regulatory subunit of phosphoinositide 3-OH kinase (PI3K) stimulating the serine-threonine kinase AKT [30]. One of the target proteins of AKT is Bad, a proapoptotic Bcl2 family member that interacts with prosurvival Bcl2 family members to promote apoptosis. Phosphorylated Bad is inactive and protects podocytes against detachment-induced cell death [30].

Combined mutations in two podocyte genes may be a common etiology for glomerular disease [67]. Crossbreeding heterozygous Cd2ap knockout mice with mice heterozygous (knockout) for synaptopodin or Fyn, which alone did not result in clinical kidney pathology, resulted in spontaneous proteinuria and FSGS-like glomerular damage supporting a role for CD2AP haploinsufficiency [60].

ACTN4

Alpha-actinin-4 (ACTN4) is an actin-bundling protein important for the integrity of the podocyte cytoskeleton associated with cell motility. Transgenic ACTN4 mice develop a severe glomerular disease and FSGS [69,70]. Lymphocytes of homozygous ACTN4 deficient mice displayed an increase in lymphocyte chemotaxis, supporting the role of ACTN4 in cell motility [70]. Podocyte cell lines derived from these ACTN4 deficient mice show less adherence to the GBM components collagen IV and laminins-10 and -11, also indicating its role in maintaining glomerular architecture and preventing disease [71].

ACTN4 is highly expressed in several human tissues. Despite the overall expression of ACTN4, the human phenotype associated with *ACTN4* mutations only manifests in the kidney. Mutations in *ACTN4* are associated with an autosomal dominant form of familial FSGS. By now, only 3 *ACTN4* missense mutations have been described in literature [72,73]. The mutated ACTN4 proteins showed a higher binding affinity to F-actin, and may change the mechanical characteristics of the podocyte [72]. *In vitro* studies in podocytes of mice homozygous for the human disease-causing K225E substitution, shows aggregation of the mutant α-actinin-4 with F-actin. Furthermore, the conformational change also leads to an increase of the actin-binding stoichiometry and a resistance to regulation by Ca^{2+} [74]. In sporadic cases of FSGS, without an apparent familial disease pattern, ACTN4 amino acid substitutions were also found [73]. However, cellular localization of the mutated proteins and actin-binding assays show that these mutations are probably not disease causing or contributing to the disease [73].

WT1

Another well described genetic defect in patients with primary nephrotic syndrome is the spectrum of clinical pictures caused by mutations in WT1 (for review 75-77), a transcription factor regulating several programs of cellular proliferation and differentiation. The *WT1* gene is positionally cloned based on its role in the development of Wilms tumors, the most common form of cancer in children [78,79]. Heterozygous *de novo* mutations in *WT1* cause Denys-Drash syndrome (DDS) and Frasier syndrome (FS), two overlapping syndromes [80], characterized by nephrotic syndrome with either diffuse mesangial sclerosis (DMS in DDS) or FSGS (in FS), genitourinary defects, and a higher risk of developing Wilms tumor (in DDS) or gonadal dysgerminoma (in both DDS and FS).

WT1 has a transcription regulating domain (encoded by exons 1 to 6) and a DNA binding zinc finger domain (encoded by exons 7 to 10). There are four major isoforms due to the insertion of the amino acids KTS between zinc fingers 3 and 4 (alternative splice site directly situated after exon 9), and due to the insertion of 17 amino acids completely encoded by exon 5. The insertion/deletion of KTS influences the space between zinc fingers 3 and 4 and thereby the binding properties of the protein [81].

The mutations found in DDS patients, but also in cases of isolated DMS, are mostly missense mutations (80%) in zinc fingers 2 and 3 (exon 8 and 9). The R394W (1180 C>T, exon 9) substitution is most frequently found in almost 50% of the DDS patients [75,82,83]. These mutations affect the DNA binding stability of WT1 to the target gene [84]. FS patients usually present with intron 9 splice site mutations affecting the +KTS/-KTS ratio [85,86]. The fact that DDS and FS are overlapping syndromes is strengthened by the finding of intron 9 splice site mutations in patients with a phenotype of DDS [80,81,87,88], and an exon 9 mutation in a patient with FS [89].

TRPC6

TRPC (transient receptor potential cation channel subfamily C, 1-7) is a subgroup of the TRP family of cation channels involved in the regulation of Ca^{2+} influx. These ion channels can be activated subsequent to either depletion of Ca^{2+} from internal stores or through receptor-mediated processes [90]. TRPC channels are expressed in many tissues. In the podocytes TRPC-1, -2, -5, and -6 are expressed [91]. TRPC6 is localized to the podocyte cell body, primary processes and in close vicinity to the slit membrane were it interacts with nephrin and podocin (not CD2AP) [91]. TRPC6 is also abundantly expressed in mesangial cells [92]. In diabetic nephropathy, the mesangial contractile function is impaired, most probably due to a reduced Ca^{2+} influx. A recent study showed that high glucose downregulates the TRPC6 protein which might contribute to the impaired Ca^{2+} signaling of mesangial cells seen in diabetes [93].

TRPC6 was found mutated in families with an autosomal dominant form of FSGS [91,94]. These mutations may cause a gain of function. The P112Q mutated TRPC6 protein showed an enhanced influx of Ca^{2+}, especially after activation of the G-protein-coupled receptor AT1 by angiotensin II, and the cellular localization of the mutant protein is more situated at the plasma membrane [94]. An increase in Ca^{2+} influx is also seen in two other missense mutations (R895C and E897K), but absent in three different ones (N143S, S270T, and K874X) [91]. In these cases an altered channel regulation or an altered interaction with other slit membrane proteins (like nephrin and podocin) may cause the disease [91]. In addition to the effects of gain-of-function mutations in the *TRPC6* gene, also elevated levels of wild-type TRPC6 protein in some acquired glomerular diseases (like membranous nephropathy and puromycin aminonucleoside (PAN)-induced albuminuria) may lead to podocyte dysfunction [95]. The pathways that TRPC6 may modulate are discussed in a recent seminar [96].

Table 1 Characteristics of hereditary diseases involved in nephrotic syndrome

List of diseases	Mode of inheritance	Protein	Protein function	Gene	Chrom.	Protein expression kidney
Congenital Nephrotic syndrome of the Finnish type (CNF)	autosomal recessive	nephrin	key component of the podocyte slit diaphragm	*NPHS1*	19q13.1	podocyte slit membrane
Steroid-Resistant Nephrotic Syndrome (SRN1)	autosomal recessive	podocin	establishment of the podocyte slit diaphragm	*NPHS2*	1q25-q31	podocyte slit membrane
familial FSGS	autosomal recessive	CD2 associated protein	cytoskeletal remodelling, cell motility, endocytosis	*CD2AP*	6	podocyte slit membrane
familial focal segmental glomerulosclerosis (FSGS1)	autosomal dominant	alpha-actinin-4	anchoring protein	*ACTN4*	19q13	predominantly podocyte cell body
Denys-Drash Syndrome (DDS) / Frasier Syndrome (FS)	*de novo* (dominant)	Wilms tumor 1	transcription factor	*WT1*	11p13	nucleus and cytoplasm of podocyte cell body
familial focal segmental glomerulosclerosis (FSGS2)	autosomal dominant	transient receptor potential cation channel 6	Ca^{2+} entry during cell proliferation	*TRPC6*	11q21-q22	tubules, podocytes, mesangial and endothelial cells
early onset familial nephrotic syndrome	autosomal recessive	phospholipase C epsilon	involved in cell growth and differentiation, gene expression	*PLCE1*	10q23	cytoplasm podocyte cell body
Pierson's syndrome	autosomal recessive	laminin β2 chain	establishment of the glomerular basement membrane	*LAMB2*	3p21	glomerular basement membrane
mitochondrial disorder	maternal	non-protein tRNA	amino acid supply	NA	mtDNA	mitochondrion renal cells
CoQ10 deficiency	autosomal recessive	parahydroxybenzo-ate-polyprenyl-transferase	electron carrier in mitochondrial respiratory chain	*COQ2*	4q21.23	mitochondrion renal cells

Table 1, continued

List of diseases	Mode of inheritance	Protein	Protein function	Gene	Chrom.	Protein expression kidney
nail-patella syndrome	*de novo* (dominant)	LIM homeobox transcription factor 1 beta	transcription factor	*LMX1B*	17q11	nucleus and cytoplasm of podocyte cell body
Schimke immuno-osseous dysplasia	autosomal recessive	SWI/SNF2-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a-like 1	gene regulation, replication, recombination, and DNA repair	*SMARCAL1*	2q34-q36	nucleus of podocyte cells and proximal tubule
mandibuloacral dysplasia	autosomal recessive	zinc metallo-proteinase STE24	potentially involved in processing of farnesylated proteins	*ZMPSTE24*	1p34	unknown
Galloway-Mowat syndrome	autosomal recessive	GMS1	unknown	*GMS1*	unknown	podocytes
Fechtner syndrome	autosomal dominant	nonmuscle myosinIIA heavy chain	actin-based motility	*MYH9*	22q12.3	tubular epithelia, mesangial cells and podocyte
action myoclonus-renal failure syndrome	autosomal recessive	lysosomal integral membrane protein type 2	lysosomal degradation of macromolecules, RNA and DNA	*SCARB2*	4q13-21	lysosomal membrane of glomerular cells (study in mice)

Chrom. = chromosome, NA = not applicable, mtDNA = mitochondrial DNA, GBM = glomerular basement membrane, FP = foot process, NS = nephrotic syndrome, ESRD = end stage renal disease, FSGS = focal segmental glomerulosclerosis, DMS = diffuse mesangial sclerosis

PLCE1

PLCE1 (phospholipase C epsilon) belongs to the phospholipase C (PLC) family involved in intracellular signaling, necessary for cell growth and differentiation. PLC plays an important role in regulating the Ca^{2+} release from internal stores as well as the influx of Ca^{2+} through cation channels (like TRPC). PLC is activated by binding of a hormone or growth factor to its receptor and PLC in turn converts phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Plasma membrane channels are activated and cations flow into the cell [97].

In the kidney, PLCE1 expression is found in the podocyte cell body and foot processes [98]. PLCE1 expression appears at the S-shaped stage of glomerular development and is highly expressed during the early capillary loop stage [98]. Recently, positional cloning identified the *PLCE1* gene being involved in families with early-onset NS and ESRD [98]. Renal histopathology generally shows DMS and histochemistry reveals reduced nephrin expression. In two patients (carrying the nontruncating missense mutation S1484L) biopsies revealed FSGS. In these patients the age of onset was relatively late as was the age of reaching ESRD [98]. A recent study in patients with isolated (non-syndromic) DMS, showed that *PLCE1* truncating mutations are more frequently found than mutations in *WT1* or *LAMB2,* two genes known to cause isolated DMS as well [99]. The authors speculate that missense mutations in *PLCE1* may be associated with a milder disease course in isolated DMS or other histologic variants such as FSGS [98,99].

A zebrafish knockdown model was used to investigate the role of PLCE1 in the maintenance of the podocyte filtration barrier during development. The zebrafish *PLCE1* ortholog was knocked-down in embryo's using antisense morpholino oligonucleotides and barrier function was assayed at 4-day-old embryo's by vascular retention of a large FITC-labeled tracer molecule. In control embryo's, the tracer molecule retained in the vasculature. Morpholino injected embryo's showed abundant FITC-positive endocytic vesicles in the pronephric tubule, distal to the glomerulus. This indicated a breakdown of the barrier function in the pronephric glomerulus [98].

Laminin β-2

The heterotrimeric laminin is assembled by three polypeptide chains: α, β, and γ. Different isoforms have been identified. Laminin-11 (α5β2γ1) is predominantly found in the adult GBM and replaces the laminin-1 (α1β1γ1) isoform, initially expressed during kidney development [100].

Mutations in the *LAMB2* gene, encoding the laminin β2 chain, are associated with the Pierson's syndrome. This syndrome is characterized by early onset nephrotic syndrome with DMS rapidly progressing to ESRD and distinct ocular abnormalities (in particular microcoria) [6]. The *LAMB2* mutations result in loss of laminin-β2 expression in the kidney and other tissues studied [6]. Mutations were also found in patients with congenital nephrotic syndrome

(and FSGS) with or without minor ocular changes [101]. These results indicate genotype-phenotype correlations among patients with *LAMB2* mutations. The authors speculated that complete loss-of-function (e.g. truncating) mutations appear to be associated with the complete Pierson syndrome, whereas missense mutations may display variable phenotypes ranging from a milder variant of Pierson's syndrome to isolated congenital nephrotic syndrome with or without minor ocular abnormalities [101]. The frequency of hematuria in these patients is not clear from the clinical data.

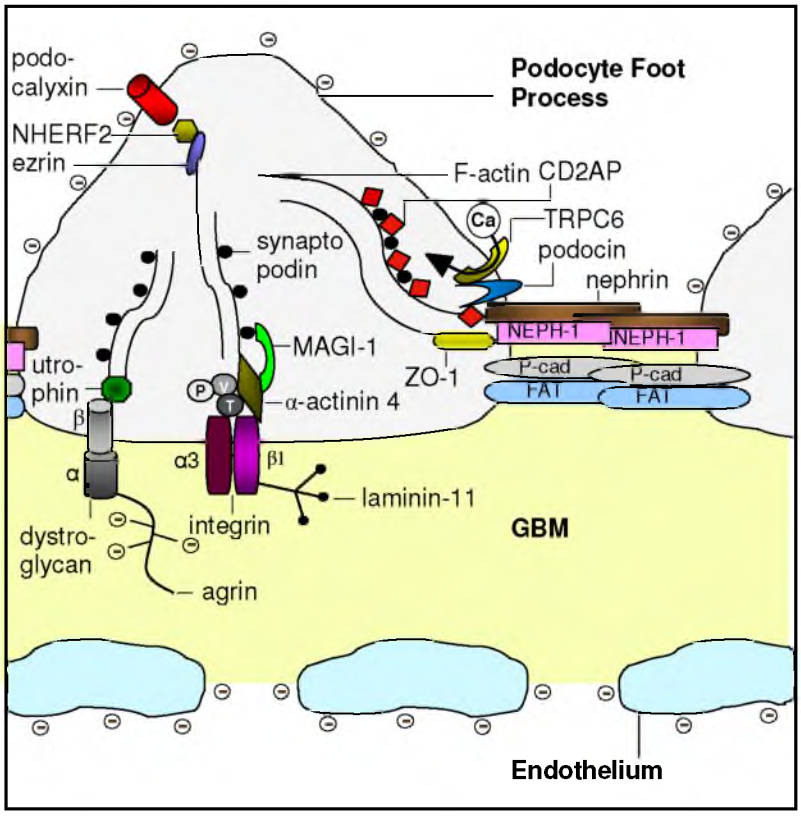

Figure 2 Molecular anatomy of the podocyte slit membrane, schematic representation. P = paxillin, V = vinculin, T = talin, Ca = calcium, ⊖ = negatively charged glycogalyx. Modified from [7], with permission.

Mitochondrial disorders

Mitochondrial cytopathies, either caused by mutations in the maternally inherited mitochondrial DNA (mtDNA) or in nuclear DNA, represent a heterogeneous group of multisystem disorders [102]. Mitochondria are non-uniformly distributed in tissues and mutated and wildtype mtDNA coexist in cells (heteroplasmy). These characteristics contribute to the large variety of clinical symptoms seen in mtDNA mutations [102].

Mutations in the tRNA $^{LEU(UUR)}$ gene are mainly associated with the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [103].

The most common transition A3243G is also found in patients with FSGS sometimes associated with maternally inherited diabetes and/or sensorineural hearing loss [104-111]. In most patients proteinuria is below nephrotic range and the FSGS progresses slowly.

The effect of the A3243G mutation to the function of the $tRNA^{LEU}$ has been studied [112]. A3243G mutant $tRNA^{Leu}$ have a shorter life span and the extent of aminoacylation ("charging" of a tRNA with an amino acid) was rather low (less than 30%) compared to wildtype [112].

Mutations were also found in other mitochondrial tRNA genes. In one patient a A5843G transition was found in the $tRNA^{Tyr}$ gene. The patient presented with mitochondrial cytopathy preceded by steroid-resistant FSGS. A skeletal muscle biopsy showed a combined respiratory chain deficiency and a partial deficiency of coenzyme Q10 most probably secondary to the oxidative damage [113]. Finally, the A4269G substitution in the $tRNA^{Ile}$ gene is described in a patient with mitochondrial encephalomyopathy and multi-organ disorders including deafness, epilepsy, FSGS, and dilated myopathy later in life [114].

Coenzyme Q10 (CoQ10) plays an important role in the electron transport from complex I and II to complex III of the mitochondrial respiratory chain. Coenzyme CoQ10 deficiency is associated with a variety of clinical phenotypes. Recently, a mutation in the nuclear DNA was found in a patient with CoQ10 deficiency [115]. This patient presented at age 12 months with proteinuria due to FSGS, mild psychomotor delay, and optic atrophy. Homozygosity mapping revealed a homozygous mutation in the *COQ2* gene, encoding *para*-hydroxybenzoate-polyprenyl transferase, an enzyme involved in the CoQ10 biosynthetic pathway [115]. The pathogenity of the mutation was demonstrated by complementation experiments in COQ2 deficient yeast and showed full functional complementation after transformation with wildtype COQ2 but not mutant COQ2 [116]. This genetic defect of the respiratory chain, is related to late-onset nephrotic syndrome with multiple organ involvement. However, respiratory chain deficiency has also been reported in one infant with congenital nephrotic syndrome characterized by diffuse mesangial hypercellularity and focal tubular dilation [117] and in one infant with glomerular lesions characterized by crescentic glomerulonephritis [118]. The encephalopathy in patients with primary CoQ10 deficiency may be improved by oral CoQ10 suppletion. Recently, it is shown that early administration of CoQ10 may also result in progressive recovery of the renal function and reduction of proteinuria [119].

Other rare cases of hereditary FSGS and recent findings

The nail-patella syndrome is a rare autosomal dominant disorder characterized by dysplasia of the nails, absent or malformed patellas, dysplasia of the elbows and frequently glaucoma and progressive nephropathy. Renal biopsies reveal non-specific findings mostly related to the degree of renal failure, including FSGS, proliferative glomerulonephritis with crescent formation, and hyalinization of the glomeruli [120]. The gene involved is the transcription

factor *LMX1B* which, among others, is required for expression of CD2AP protein and podocin [121].

Mutations in *SMARCAL1* are involved in the development of Schimke immuno-osseous dysplasia. This autosomal recessive disorder is characterized by spondyloepiphyseal dysplasia causing growth retardation, defective cellular immunity, hyperpigmented macules, dysmorphic facial feature and nephrotic syndrome (due to FSGS). *SMARCAL1* encodes an actin dependent regulator of chromatin which is involved in gene regulation, replication, recombination, and DNA repair [122]. More detailed studies of its role are provided by Elizondo *et al* [123]. Recently, mutations in *SMARCAL1* were also found in two siblings with an *incomplete* phenotype of Schimke immuno-osseous dysplasia. The siblings were initially classified as suffering from familial steroid-resistant nephrotic syndrome. In prepuberty they had proportionate short stature which developed into disproportions during adolescence. No other syndrome-specific symptoms were found. Milder phenotypes may be clinically overlooked [124]. It has been demonstrated that anthropometric measures are helpful to distinguish Schimke immuno-osseous dysplasia from other forms of chronic kidney disease [125].

In three patients (of two families) a nonsense mutation in tetraspanin CD151 causes end-stage familial nephropathy with pretibial epidermolysis bullosa and sensorineural deafness. The only available renal biopsy of one patient did not show FSGS but splitting of the tubular basement membrane and thickening and fragmentation of the GBM [126]. CD151 null mice, however, develop massive proteinuria while aging caused by FSGS [127].

Mandibuloacral dysplasia (MAD) is a rare autosomal recessive syndrome with variable clinical features. These features include mandibular and clavicular hypoplasia, acro-osteolysis of terminal phalanges, delayed closure of cranial sutures, joint contractures, mottled pigmentation and lipodystrophy. MAD is also genetically heterogeneous: two loci have been identified. So far, most mutations were found in *LMNA*, encoding lamin A, a structural protein component of the nuclear lamina determining nuclear shape and size [128]. Only three patients have been reported with compound heterozygous mutations in *ZMPSTE24*. This gene encodes a zinc metalloproteinase involved in post-translational processing of prelamin A. Two of them presented with renal failure caused by FSGS, suggesting FSGS as a phenotypic manifestation in patients with ZMPSTE24 deficiency [129].

The Galloway-Mowat syndrome (GMS) is an autosomal recessive disorder characterized by microcephaly, severe mental retardation, hiatal hernia, and steroid-resistant nephrotic syndrome. In GMS, the nephrotic syndrome occurs in the first four months of life, is steroid-resistant and rapidly progresses to end stage renal disease. Histology shows heterogeneous lesions: minimal changes, endocapillary proliferation, FSGS (in most cases), and, in end stages, DMS [130]. Recent linkage studies in two Algerian families identified a homozygous mutation in the *GMS1* gene. The protein encoded by this gene is expressed inmany tissues, including brain and glomerular podocytes, and has yet an unknown function [Nevo F, Machuca EA, Gribouval O *et al* (2008) Abstract J Am Soc Nephrol].

Recently, *MYH9* and *SCARB2* have been identified as being involved in the development of FSGS. *MYH9* encodes for the heavy chain of nonmuscle myosinIIA (NMMHC-IIA). Myosins of class II are widely distributed in most tissues and are essential components of the cell motor system involved in several important cell functions. These, among others, include phagocytosis, maintenance of cell shape and polarity, and intracellular organelle/particle trafficking [131]. Nonmuscle myosinII is composed of two heavy chains and two pairs of light chains. The NH2-terminal region contains an actin and ATP binding domain required for motor activity. The COOH-terminus allows the molecules to form filaments [132]. In the kidney, NMMHC-IIA is localized in the tubular epithelia and in the glomeruli it is expressed in podocytes and mesangial cells [133].

Heterozygous mutations of *MYH9* are involved in a complex disorder named *MYH9*-related disease characterized by platelet macrocytosis, thrombocytopenia and leukocyte inclusions containing NMMHC-IIA. Complications that may arise are deafness, cataracts and renal failure. In the past, these patients were classified as being affected by May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome or Epstein syndrome. It is now believed that these disorders are not distinct entities, but rather a single illness with a continuous clinical spectrum [131]. In a large family with Fechtner syndrome, ten members carried a *MYH9* missense mutation. Only two members had renal problems. Electron microscopy showed FSGS and segmental effacement of podocytes in these patients [133]. Why the other family members did not develop renal problems remains unclear. Most probably other additional factors play a role [133, 134]. More recently, *MYH9* was suggested to be a risk gene for the development of idiopathic FSGS by usage of a genome scan on African-American individuals with FSGS. However, sequencing of the 40 exons and intron-exon junctions of *MYH9* did not reveal any causal sequence variation. The authors hypothesize that the variation may occur in regulatory elements or splice-site determinants influencing RNA expression or protein structure [134]. Future studies are necessary to identify the precise role of *MYH9* in the development of FSGS.

Action myoclonus-renal failure (AMRF) syndrome is a lethal inherited form of progressive myoclonus epilepsy associated with renal failure due to FSGS. It usually starts at 15-25 years of age with proteinuria or neurological symptoms as tremor, action myoclonus or seizures associated with storage material in the brain [135]. Mutations in *SCARB2* were found in three families with a single AMRF proband [135,136]. However, analysis of patients with nonsyndromic FSGS, MCNS, thin-basement membrane nephropathy, or non-Alport with haematuria and proteinuria did not reveal any mutations indicating a specific role of *SCARB2* in AMRF [135]. *SCARB2* encodes for the ubiquitously expressed lysosomal integral membrane protein type 2 (LIMP-2) mainly found in lysosomes and late endosomes [136]. This LIMP-2 protein has been shown to act as a receptor to bind β-glucocerebrosidase, the enzyme defective in Gaucher disease (lysosomal storage disorder) [137]. LIMP-2 deficient mice have also a kidney phenotype. The kidneys, which are non-hydronephrotic show pelvi-ureteric obstruction and glomerular lesions with mesangial hypercellularity and effacement of foot processes [135].

Outline of the thesis

In the last years, the genetic defects described above, gave insight in the structure and function of the podocyte slit membrane and the development of nephrotic syndrome. FSGS patients belong to a heterogeneous group with different underlying causes. Although the development of FSGS is not completely and solely explained by these genetic defects, the knowledge whether a FSGS patient carries a genetic defect turned out to be very important for the treatment modality and prognosis of the patient. For example, most of the patients with *NPHS2* mutations are steroid-resistant and further treatment with steroids may be stopped. Also, in most cases, there is no recurrence of nephrotic syndrome after renal transplantation in these patients. In patients carrying *WT1* mutations further medical examination and regular check-ups are important given the higher risk of developing malignancies in these patients. Finally, mtDNA mutations may cause multi-systemic disorders.

The aim of this thesis was to further elucidate the role of podocyte dysfunction in the development of FSGS. In *Chapter 2* we describe 2 patients with idiopathic steroid-resistant FSGS due to mutations in the *WT1* and *NPHS2* gene and we discuss the necessity of providing a standard mutation analysis protocol for steroid-resistant FSGS patients.

In *Chapter 3* a patient is presented with the A3243G transition, particularly associated with the MELAS syndrome. This patient, in contrast to other described cases, presented with a full-blown nephrotic syndrome caused by FSGS. Physicians should be mindful for mitochondrial abnormalities, especially when there is a family history of diabetes mellitus, hearing loss, or neuromuscular disorders.

Chapter 4 describes a patient who, at later age than usual, was diagnosed with the Denys-Drash syndrome due to a mutation in the *WT1* gene. The case is presented to inform physicians, other than paediatricians, about the syndrome and its treatment.

The effect of a *CD2AP* mutation on its interaction with F-actin is described in *Chapter 5*. We describe a patient with a homozygous mutation in the *CD2AP* gene and by *in vitro* studies we showed a decreased interaction between F-actin and the mutated protein. Furthermore, isolated lymphocytes of the patient show no CD2AP expression.

An overview of mutations found in patients with idiopathic steroid-resistant FSGS is shown in *Chapter 6*. Moreover the role of combined gene defects is discussed.

Long-term consequences and the role of podocyte gene defects in the development of MCNS is evaluated in *Chapter 7*. Limited genetic abnormalities mutations were found in this group of patients suggesting these podocyte genes do not play a major role in the pathophysiology of MCNS.

To investigate the role of agrin, a GBM heparan sulfate proteoglycan, in the glomerular filtration system, we tried to develop a kidney-specific agrin knockout mouse model. These results are described in *Chapter 8*.

The results of these studies are summarized, discussed and put into perspective in *Chapter 9*.

References

1. Salmon AH, Neal CR, Harper SJ. New aspects of glomerular filtration barrier structure and function: five layers (at least) not three. *Curr Opin Nephrol Hypertens*. 2009; **18:** 197-205.

2. Ballermann BJ. Glomerular endothelial cell differentiation. *Kidney Int*. 2005; **67:** 1668-1671.

3. Jeansson M, Haraldsson B. Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. *Am J Physiol Renal Physiol*. 2006; **290:** F111-116.

4. Levidiotis V, Power DA. New insights into the molecular biology of the glomerular filtration barrier and associated disease. *Nephrology (Carlton)*. 2005; **10:** 157-166.

5. Barker DF, Hostikka SL, Zhou J *et al*. Identification of mutations in the COL4A5 collagen gene in Alport syndrome. *Science*. 1990; **248:** 1224-1227.

6. Zenker M, Aigner T, Wendler O *et al*. Human laminin beta2 deficiency causes congenital nephrosis with mesangial sclerosis and distinct eye abnormalities. *Hum Mol Genet*. 2004; **13:** 2625-2632.

7. Pavenstädt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev*. 2003; **83:** 253-307.

8. Kretzler M. Regulation of adhesive interaction between podocytes and glomerular basement membrane. *Microsc Res Tech*. 2002; **57:** 247-253.

9. Rodewald R, Karnovsky MJ. Porous substructure of the glomerular slit diaphragm in the rat and mouse. *J Cell Biol*. 1974; **60:** 423-433.

10. Neal CR, Crook H, Bell E *et al*. Three-dimensional reconstruction of glomeruli by electron microscopy reveals a distinct restrictive urinary subpodocyte space. *J Am Soc Nephrol*. 2005; **16:** 1223-1235.

11. McTaggart SJ. Childhood urinary conditions. *Aust Fam Physician*. 2005; **34:** 937-941.

12. Srivastava T, Simon SD, Alon US. High incidence of focal segmental glomerulosclerosis in nephrotic syndrome of childhood. *Pediatr Nephrol*. 1999; **13:** 13-18.

13. Woroniecki RP, Kopp JB. Genetics of focal segmental glomerulosclerosis. *Pediatr Nephrol*. 2007; **22:** 638-644.

14. D'Agati V, Pathologic classification of focal segmental glomerulosclerosis. *Semin Nephrol*. 2003; **23:** 117-134.

15. D'Agati VD, Fogo AB, Bruijn JA, Jennette JC. Pathologic classification of focal segmental glomerulosclerosis: a working proposal. *Am J Kidney Dis*. 2004; **43:** 368-382.

16. D'Agati VD. The spectrum of focal segmental glomerulosclerosis: new insights. *Curr Opin Nephrol Hypertens*. 2008; **17:** 271-281.

17. Thomas DB, Franceschini N, Hogan SL *et al*. Clinical and pathologic characteristics of focal segmental glomerulosclerosis pathologic variants. *Kidney Int*. 2006; **69:** 920-926.

18. Artero M, Biava C, Amend W *et al*. Recurrent focal glomerulosclerosis: natural history and response to therapy. *Am J Med*. 1992; **92:** 375-383.

19. Dall'Amico R, Ghiggeri G, Carraro M *et al*. Prediction and treatment of recurrent focal segmental glomerulosclerosis after renal transplantation in children. *Am J Kidney Dis*. 1999; **34:** 1048-1055.

20. Savin VJ, Sharma R, Sharma M *et al*. Circulating factor associated with increased glomerular permeability to albumin in recurrent focal segmental glomerulosclerosis. *N Engl J Med*. 1996; **334:** 878-883.

21. Artero ML, Sharma R, Savin VJ, Vincenti F. Plasmapheresis reduces proteinuria and serum capacity to injure glomeruli in patients with recurrent focal glomerulosclerosis. *Am J Kidney Dis.* 1994; **23:** 574-581.

22. Kershaw DB, Sedman AB, Kelsch RC, Bunchman TE. Recurrent focal segmental glomerulosclerosis in pediatric renal transplant recipients: successful treatment with oral cyclophosphamide. *Clin Transplant.* 1994; **8:** 546-549.

23. Hattori M, Akioka Y, Chikamoto H *et al.* Increase of integrin-linked kinase activity in cultured podocytes upon stimulation with plasma from patients with recurrent FSGS. *Am J Transplant.* 2008; **8:** 1550-1556.

24. Savin VJ, Sharma M, McCarthy ET *et al.* Cardiotrophin like cytokine-1: candidate for the focal segmental sclerosis permeability factor. *J Am Soc Nephrol.* 2008; **19:** 59A (F-FC260).

25. Ilagan MX, Kopan R. SnapShot: Notch signaling pathway. *Cell.* 2007; **128:** 1246.

26. Vooijs M, Ong CT, Hadland B *et al.* Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE. *Development.* 2007; **134:** 535-544.

27. Niranjan T, Bielesz B, Gruenwald A *et al.* The Notch pathway in podocytes plays a role in the development of glomerular disease. *Nat Med.* 2008; **14:** 290-298.

28. Putaala H, Soininen R, Kilpelainen P *et al.* The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. *Hum Mol Genet.* 2001; **10:** 1-8.

29. Orikasa M, Matsui K, Oite T, Shimizu F. Massive proteinuria induced in rats by a single intravenous injection of a monoclonal antibody. *J Immunol.* 1988; **141:** 807-814.

30. Huber TB, Hartleben B, Kim J *et al.* Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol Cell Biol.* 2003; **23:** 4917-4928.

31. Huber TB, Kottgen M, Schilling B *et al.* Interaction with podocin facilitates nephrin signaling. *J Biol Chem.* 2001; **276:** 41543-41546.

32. Simons M, Schwarz K, Kriz W *et al.* Involvement of lipid rafts in nephrin phosphorylation and organization of the glomerular slit diaphragm. *Am J Pathol.* 2001; **159:** 1069-1077.

33. Barisoni L, Kopp JB. Update in podocyte biology: putting one's best foot forward. *Curr Opin Nephrol Hypertens.* 2003; **12:** 251-258.

34. Verma R, Wharram B, Kovari I *et al.* Fyn binds to and phosphorylates the kidney slit diaphragm component Nephrin. *J Biol Chem.* 2003; **278:** 20716-20723.

35. Kestila M, Lenkkeri U, Mannikko M *et al.* Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome. *Mol Cell.* 1998; **1:** 575-582.

36. Patrakka J, Ruotsalainen V, Reponen P *et al.* Recurrence of nephrotic syndrome in kidney grafts of patients with congenital nephrotic syndrome of the Finnish type: role of nephrin. *Transplantation.* 2002; **73:** 394-403.

37. Wang SX, Ahola H, Palmen T *et al.* Recurrence of nephrotic syndrome after transplantation in CNF is due to autoantibodies to nephrin. *Exp Nephrol.* 2001; **9:** 327-331.

38. Laakkonen H, Lönnqvist T, Uusimaa J *et al.* Muscular dystonia and athetosis in six patients with congenital nephrotic syndrome of the Finnish type (NPHS1). *Pediatr Nephrol.* 2006; **21:** 182-189.

39. Boute N, Gribouval O, Roselli S *et al.* NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet.* 2000; **24:** 349-354.

40. Roselli S, Gribouval O, Boute N *et al.* Podocin localizes in the kidney to the slit diaphragm area. *Am J Pathol.* 2002; **160:** 131-139.

41. Schwarz K, Simons M, Reiser J *et al.* Podocin, a raft-associated component of the glomerular slit diaphragm interacts with CD2AP and nephrin. *J Clin Invest.* 2001; **108:** 1621-1629.

42. Sellin L, Huber TB, Gerke P *et al.* NEPH1 defines a novel family of podocin interacting proteins. *FASEB J.* 2003; **17:** 115-117.

43. Liu XL, Done SC, Yan K *et al.* Defective trafficking of nephrin missense mutants rescued by a chemical chaperone. *J Am Soc Nephrol.* 2004; **15:** 1731-1738.

44. Roselli S, Heidet L, Sich M *et al.* Early glomerular filtration defect and severe renal disease in podocin-deficient mice. *Mol Cell Biol.* 2004; **24:** 550-560.

45. Koziell A, Grech V, Hussain S *et al.* Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. *Hum Mol Genet.* 2002; **11:** 379-388.

46. Karle SM, Uetz B, Ronner V *et al.* Novel mutations in NPHS2 detected in both familial and sporadic steroid-resistant nephrotic syndrome. *J Am Soc Nephrol.* 2002; **13:** 388-393.

47. Tsukaguchi H, Sudhakar A, Le TC. NPHS2 mutations in late-onset focal segmental glomerulosclerosis: R229Q is a common disease-associated allele. *J Clin Invest.* 2002; **110:** 1659-1666.

48. Caridi G, Bertelli R, Scolari F. Podocin mutations in sporadic focal-segmental glomerulosclerosis occurring in adulthood. *Kidney Int.* 2004; **64:** 365.

49. Philippe A, Nevo F, Esquivel EL, Reklaityte D *et al.* Nephrin mutations can cause childhood-onset steroid-resistant nephrotic syndrome. *J Am Soc Nephrol.* 2008; **19:** 1871-1878.

50. Pereira AC, Pereira AB, Mota GF *et al.* NPHS2 R229Q functional variant is associated with micro-albuminuria in the general population. *Kidney Int.* 2004; **65:** 1026-1030.

51. Huber TB, Simons M, Hartleben B *et al.* Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. Hum Mol Genet. 2003; 12: 3397-3405.

52. Roselli S, Moutkine I, Gribouval O *et al.* Plasma membrane targeting of podocin through the classical exocytic pathway: effect of NPHS2 mutations. *Traffic.* 2004; **5:** 37-44.

53. Nishibori Y, Liu L, Hosoyamada M *et al.* Disease-causing missense mutations in NPHS2 gene alter normal nephrin trafficking to the plasma membrane. *Kidney Int.* 2004; **66:** 1755-1765.

54. Bertelli R, Ginevri F, Caridi G *et al.* Recurrence of focal segmental glomerulosclerosis after renal transplantation in patients with mutations of podocin. *Am J Kidney Dis.* 2003; **41:** 1314-1321.

55. Ruf RG, Lichtenberger A, Karle SM *et al.* Patients with mutations in NPHS2 (podocin) do not respond to standard steroid treatment of nephrotic syndrome. *J Am Soc Nephrol.* 2004; **15:** 722-732.

56. Weber S, Gribouval O, Esquivel EL *et al.* NPHS2 mutation analysis shows genetic heterogeneity of steroid-resistant nephrotic syndrome and low post-transplant recurrence. *Kidney Int.* 2004; **66:** 571-579.

57. Caridi G, Perfumo F, Ghiggeri GM. NPHS2 (Podocin) mutations in nephrotic syndrome. Clinical spectrum and fine mechanisms. *Pediatr Res.* 2005; **57:** 54R-61R.

58. Dustin ML, Olszowy MW, Holdorf AD *et al.* A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell.* 1998; **94:** 667-677.

59. Shih NY, Li J, Karpitskii V *et al.* Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science.* 1999; **286:** 312-315.

60. Kim JM, Wu H, Green G *et al.* CD2-associated protein haplo-insufficiency is linked to glomerular disease susceptibility. *Science.* 2003; **300:** 1298-1300.

61. Kirsch KH, Georgescu MM, Ishimaru S, Hanafusa H. CMS: an adapter molecule involved in cytoskeletal rearrangements. *Proc Natl Acad Sci U S* A. 1999; **96:** 6211-6216.

62. Schiffer M, Mundel P, Shaw AS, Böttinger EP. A novel role for the adaptor molecule CD2-associated protein in transforming growth factor-beta-induced apoptosis. *J Biol Chem.* 2004; **279:** 37004-37012.

63. Cormont M, Meton I, Mari M *et al.* CD2AP/CMS regulates endosome morphology and traffic to the degradative pathway through its interaction with Rab4 and c-Cbl. *Traffic.* 2003; **4:** 97-112.

64. Lynch DK, Winata SC, Lyons RJ *et al.* A cortactin-CD2-associated protein (CD2AP) complex provides a novel link between epidermal growth factor receptor endocytosis and the actin cytoskeleton. *J Biol Chem.* 2003; **278:** 21805-21813.

65. Kobayashi S, Sawano A, Nojima Y *et al.* The c-Cbl/CD2AP complex regulates VEGF-induced endocytosis and degradation of Flt-1 (VEGFR-1). *FASEB J.* 2004; **18:** 929-931.

66. Lehtonen S, Zhao F, Lehtonen E. CD2-associated protein directly interacts with the actin cytoskeleton. *Am J Physiol Renal Physiol.* 2002; **283:** F734-743.

67. Huber TB, Kwoh C, Wu H *et al.* Bigenic mouse models of focal segmental glomerulosclerosis involving pairwise interaction of CD2AP, Fyn, and synaptopodin. *J Clin Invest.* 2006; **116:** 1337-1345.

68. Shih NY, Li J, Cotran R *et al.* CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am J Pathol.* 2001; **159:** 2303-2308.

69. Michaud JL, Lemieux LI, Dube M *et al.* Focal and segmental glomerulosclerosis in mice with podocyte-specific expression of mutant alpha-actinin-4. *J Am Soc Nephrol.* 2003; **14:** 1200-1211.

70. Kos CH, Le TC, Sinha S *et al.* Mice deficient in alpha-actinin-4 have severe glomerular disease. J *Clin Invest.* 2003; **111:** 1683-1690.

71. Dandapani SV, Sugimoto H, Matthews BD *et al.* Alpha-actinin-4 is required for normal podocyte adhesion. *J Biol Chem.* 2007; **282:** 467-477.

72. Kaplan JM, Kim SH, North KN *et al.* Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet.* 2000; **24:** 251-256.

73. Weins A, Kenlan P, Herbert S *et al.* Mutational and biological analysis of alpha-actinin-4 in focal segmental glomerulosclerosis. *J Am Soc Nephrol.* 2005; **16:** 3694-3701.

74. Weins A, Schlondorff J, Nakamura F *et al.* Disease-associated mutant alpha-actinin-4 reveals a mechanism for regulating its F-actin-binding affinity. *Proc Natl Acad Sci U S A.* 2007; **104:** 16080-16085.

75. Little M, Wells C. A clinical overview of WT1 gene mutations. *Hum Mutat.* 1997; **9:** 209-225.

76. Wagner KD, Wagner N, Schedl A. The complex life of WT1. *J Cell Sci.* 2003; **116:** 1653-1658.

77. Morrison AA, Viney RL, Saleem MA, Ladomery MR. New insights into the function of the Wilms tumor suppressor gene WT1 in podocytes. *Am J Physiol Renal Physiol.* 2008; **295:** F12-F17.

78. Haber DA, Buckler AJ, Glaser T *et al.* An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. *Cell.* 1990; **61:** 1257-1269.

79. Gessler M, Poustka A, Cavenee W *et al.* Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature.* 1990; **343:** 774-778.

80. McTaggart SJ, Algar E, Chow CW *et al.* Clinical spectrum of Denys-Drash and Frasier syndrome. *Pediatr Nephrol.* 2001; **16:** 335-339.

81. Haber DA, Sohn RL, Buckler AJ. Alternative splicing and genomic structure of the Wilms tumor gene WT1. *Proc Natl Acad Sci U S A.* 1991; **88:** 9618-9622.

82. Pelletier J, Bruening W, Kashtan CE *et al.* Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell.* 1991; **67:** 437-447.

83. Bruening W, Bardeesy N, Silverman BL *et al.* Germline intronic and exonic mutations in the Wilms' tumour gene (WT1) affecting urogenital development. *Nat Genet.* 1992; 1**:** 144-148.

84. Little M, Holmes G, Bickmore W *et al.* DNA binding capacity of the WT1 protein is abolished by Denys-Drash syndrome WT1 point mutations. *Hum Mol Genet.* 1995; **4:** 351-358.

85. Barbaux S, Niaudet P, Gubler MC *et al.* Donor splice-site mutations in WT1 are responsible for Frasier syndrome. *Nat Genet.* 1997; **17:** 467-470.

86. Klamt B, Koziell A, Poulat F *et al.* Frasier syndrome is caused by defective alternative splicing of WT1 leading to an altered ratio of WT1 +/-KTS splice isoforms. *Hum Mol Genet.* 1998; **7:** 709-714.

87. Denamur E, Bocquet N, Mougenot B *et al.* Mother-to-child transmitted WT1 splice-site mutation is responsible for distinct glomerular diseases. *J Am Soc Nephrol.* 1999; **10:** 2219-2223.

88. Jeanpierre C, Denamur E, Henry I *et al.* Identification of constitutional WT1 mutations, in patients with isolated diffuse mesangial sclerosis, and analysis of genotype/phenotype correlations by use of a computerized mutation database. *Am J Hum Genet.* 1998; **62:** 824-833.

89. Kohsaka T, Tagawa M, Takekoshi Y *et al.* Exon 9 mutations in the WT1 gene, without influencing KTS splice isoforms, are also responsible for Frasier syndrome. *Hum Mutat.* 1999; **14:** 466-470.

90. Riccio A, Medhurst AD, Mattei C *et al.* mRNA distribution analysis of human TRPC family in CNS and peripheral tissues. *Brain Res Mol Brain Res.* 2002; **109:** 95-104.

91. Reiser J, Polu KR, Moller CC *et al.* TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. *Nat Genet.* 2005; **37:** 739-744.

92. Sours S, Du J, Chu S *et al.* Expression of canonical transient receptor potential (TRPC) proteins in human glomerular mesangial cells. *Am J Physiol Renal Physiol.* 2006; **290:** F1507-F1515.

93. Graham S, Ding M, Sours-Brothers S *et al.* Downregulation of TRPC6 protein expression by high glucose, a possible mechanism for the impaired Ca2+ signaling in glomerular mesangial cells in diabetes. *Am J Physiol Renal Physiol.* 2007; **293:** F1381-F1390.

94. Winn MP, Conlon PJ, Lynn KL *et al.* A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science.* 2005; **308:** 1801-1804.

95. Moller CC, Wei C, Altintas MM *et al.* Induction of TRPC6 channel in acquired forms of proteinuric kidney disease. *J Am Soc Nephrol.* 2007; **18:** 29-36.

96. Schlöndorff JS, Pollak MR. TRPC6 in glomerular health and disease: what we know and what we believe. Semin Cell Dev Biol. 2006; **17:** 667-674.

97. Freichel M, Vennekens R, Olausson J *et al.* Functional role of TRPC proteins in native systems: implications from knockout and knock-down studies. *J Physiol.* 2005; **567:** 59-66.

98. Hinkes B, Wiggins RC, Gbadegesin R *et al.* Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. *Nat Genet.* 2006; **38:** 1397-1405.

99. Gbadegesin R, Hinkes BG, Hoskins BE *et al.* Mutations in PLCE1 are a major cause of isolated diffuse mesangial sclerosis (IDMS). *Nephrol Dial Transplant.* 2008; **23:** 1291-1297.

100. St John PL, Abrahamson DR. Glomerular endothelial cells and podocytes jointly synthesize laminin-1 and -11 chains. *Kidney Int.* 2001; **60:** 1037-1046.

101. Hasselbacher K, Wiggins RC, Matejas V *et al.* Recessive missense mutations in LAMB2 expand the clinical spectrum of LAMB2-associated disorders. *Kidney Int.* 2006; **70:** 1008-1012.

102. Schmiedel J, Jackson S, Schafer J, Reichmann H. Mitochondrial cytopathies. *J Neurol.* 2003; **250:** 267-277.

103. Piechota J, Mroczek K, Bartnik E. MELAS as an example of a mitochondrial disease. *J Appl Genet.* 2001; **42:** 351-358.

104. Jansen JJ, Maassen JA, van der Woude FJ *et al.* Mutation in mitochondrial tRNA(Leu(UUR)) gene associated with progressive kidney disease. *J Am Soc Nephrol.* 1997; **8:** 1118-1124.

105. Kurogouchi F, Oguchi T, Mawatari E *et al.* A case of mitochondrial cytopathy with a typical point mutation for MELAS, presenting with severe focal segmental glomerulosclerosis as main clinical manifestation. *Am J Nephrol.* 1998; **18:** 551-556.

106. Nakamura S, Yoshinari M, Doi Y *et al.* Renal complications in patients with diabetes mellitus associated with an A to G mutation of mitochondrial DNA at the 3243 position of leucine tRNA. *Diabetes Res Clin Pract.*1999; **44:** 183-189.

107. Cheong HI, Chae JH, Kim JS *et al.* Hereditary glomerulopathy associated with a mitochondrial tRNA(Leu) gene mutation. *Pediatr Nephrol.* 1999; **13:** 477-480.

108. Doleris LM, Hill GS, Chedin P *et al.* Focal segmental glomerulosclerosis associated with mitochondrial cytopathy. *Kidney Int.* 2000; **58:** 1851-1858.

109. Hotta O, Inoue CN, Miyabayashi S. Clinical and pathologic features of focal segmental glomerulosclerosis with mitochondrial tRNALeu(UUR) gene mutation. *Kidney Int.* 2001; **59:** 1236-1243.

110. Yamagata K, Muro K, Usui J *et al.* Mitochondrial DNA mutations in focal segmental glomerulosclerosis lesions. *J Am Soc Nephrol.* 2002; **13:** 1816-1823.

111. Guery B, Choukroun G, Noel LH *et al.* The spectrum of systemic involvement in adults presenting with renal lesion and mitochondrial tRNA (Leu) gene mutation. *J Am Soc Nephrol.* 2003; **14:** 2099-2108.

112. Yasukawa T, Suzuki T, Ueda T *et al.* Modification defect at anticodon wobble nucleotide of mitochondrial tRNAs(Leu)(UUR) with pathogenic mutations of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. *J Biol Chem.* 2000; **275:** 4251-4257.

113. Scaglia F, Vogel H, Hawkins EP *et al.* Novel homoplasmic mutation in the mitochondrial tRNATyr gene associated with atypical mitochondrial cytopathy presenting with focal segmental glomerulosclerosis. *Am J Med Genet A.* 2003; **123:** 172-178.

114. Taniike M, Fukushima H, Yanagihara I *et al.* Mitochondrial tRNA(Ile) mutation in fatal cardiomyopathy. *Biochem Biophys Res Commun.* 1992; **15:** 47-53.

115. Quinzii C, Naini A, Salviati L *et al.* A mutation in para-hydroxybenzoate-polyprenyl transferase (COQ2) causes primary coenzyme Q10 deficiency. *Am J Hum Genet.* 2006; **78:** 345-349.

116. Lopez-Martin JM, Salviati L, Trevisson E *et al.* Missense mutation of the COQ2 gene causes defects of bioenergetics and de novo pyrimidine synthesis. *Hum Mol Genet.* 2007; **16:** 1091-1097.

117. Goldenberg A, Ngoc LH, Thouret MC *et al.* Respiratory chain deficiency presenting as congenital nephrotic syndrome. *Pediatr Nephrol.* 2005; **20:** 465-469.

118. Diomedi-Camassei F, Di Giandomenico S, Santorelli FM *et al.* COQ2 nephropathy: a newly described inherited mitochondriopathy with primary renal involvement. *J Am Soc Nephrol.* 2007; **18:** 2773-2780.

119. Montini G, Malaventura C, Salviati L. Early coenzyme Q10 supplementation in primary coenzyme Q10 deficiency. *N Engl J Med.* 2008; **358:** 2849-2850.

120. Bongers EM, Gubler MC, Knoers NV. Nail-patella syndrome. Overview on clinical and molecular findings. *Pediatr Nephrol.* 2002; **17:** 703-712.

121. Miner JH, Morello R, Andrews KL *et al.* Transcriptional induction of slit diaphragm genes by Lmx1b is required in podocyte differentiation. *J Clin Invest.* 2002; **109:** 1065-1072.

122. Boerkoel CF, Takashima H, John J *et al.* Mutant chromatin remodeling protein SMARCAL1 causes Schimke immuno-osseous dysplasia. *Nat Genet.* 2002; **30:** 215-220.

123. Elizondo LI, Cho KS, Zhang W *et al.* Schimke immuno-osseous dysplasia: SMARCAL1 loss-of-function and phenotype correlation. *J Med Genet.* 2009; **46:** 49-59.

124. Zivičnjak M, Franke D, Zenker M *et al.* SMARCAL1 mutations: A cause of prepubertal idiopathic steroid-resistant nephrotic syndrome. *Pediatr Res.* 2009; **65:** 564-568.

125. Lücke T, Franke D, Clewing JM *et al.* Schimke versus non-Schimke chronic kidney disease: an anthropometric approach. *Pediatrics.* 2006; **118:** e400-407.

126. Karamatic Crew V, Poole J, Long S *et al.* Two MER2-negative individuals with the same novel CD151 mutation and evidence for clinical significance of anti-MER2. *Transfusion.* 2008; **48:** 1912-1916.

127. Sachs N, Kreft M, van den Bergh Weerman MA *et al.* Kidney failure in mice lacking the tetraspanin CD151. *J Cell Biol.* 2006; **175:** 33-39.

128. Jacob KN, Garg A. Laminopathies: multisystem dystrophy syndromes. *Mol Genet Metab.* 2006; **87:** 289-302.

129. Agarwal AK, Zhou XJ, Hall RK *et al.* Focal segmental glomerulosclerosis in patients with mandibuloacral dysplasia owing to ZMPSTE24 deficiency. *J Investig Med.* 2006; **54:** 208-13.

130. Sartelet H, Pietrement C, Noel LH *et al.* Collapsing glomerulopathy in Galloway-Mowat syndrome: a case report and review of the literature. *Pathol Res Pract.* 2008; **204:** 401-406.

131. Marini M, Bruschi M, Pecci A *et al.* Non-muscle myosin heavy chain IIA and IIB interact and co-localize in living cells: relevance for MYH9-related disease. *Int J Mol Med.* 2006; **17:** 729-736.

132. Seri M, Savino M, Bordo D *et al.* Epstein syndrome: another renal disorder with mutations in the nonmuscle myosin heavy chain 9 gene. *Hum Genet.* 2002; **110:** 182-186.

133. Ghiggeri GM, Caridi G, Magrini U *et al.* Genetics, clinical and pathological features of glomerulonephritis associated with mutations of nonmuscle myosin IIA (Fechtner syndrome). *Am J Kidney Dis.* 2003; **41:** 95-104.

134. Kopp JB, Smith MW, Nelson GW *et al.* MYH9 is a major-effect risk gene for focal segmental glomerulosclerosis. *Nat Genet.* 2008; **40:** 1175-1184.

135. Berkovic SF, Dibbens LM, Oshlack A *et al.* Array-based gene discovery with three unrelated subjects shows SCARB2/LIMP-2 deficiency causes myoclonus epilepsy and glomerulosclerosis. *Am J Hum Genet.* 2008; **82:** 673-684.

136. Balreira A, Gaspar P, Caiola D *et al.* A nonsense mutation in the LIMP-2 gene associated with progressive myoclonic epilepsy and nephrotic syndrome. *Hum Mol Genet.* 2008; **17:** 2238-2243.

137. Reczek D, Schwake M, Schröder J *et al.* LIMP-2 is a receptor for lysosomal mannose-6-phosphate-independent targeting of beta-glucocerebrosidase. *Cell.* 2007; **131:** 770-783.

Chapter 2

WT-1 and *NPHS2* mutation analysis in patients with non-familial steroid-resistant focal segmental glomerulosclerosis

Familial forms of steroid-resistant nephrotic syndrome with the histologic findings of focal segmental glomerulosclerosis have frequently a genetic basis. For the non-familial forms this is still unresolved. Ten children with non-familial steroid-resistant nephrotic syndrome along with focal segmental glomerulosclerosis were tested for mutations in the *WT-1* and *NPHS2* genes. In one patient a mutation in intron 9 of the *WT-1* gene and in one patient a heterozygous *NPHS2* mutation could be detected. Both abnormalities are important for the treatment modalities and prognosis. Additional studies will have to provide a solid basis for the recommendation of mutation analysis in non-familial steroid-resistant focal segmental glomerulosclerosis.

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Introduction

Much progress has been made in the genetic classification of familial steroid-resistant nephrotic syndrome with the histologic findings of focal segmental glomerulosclerosis (FSGS).

An autosomal recessive form is due to mutations within the *NPHS2* gene, encoding podocin, a new integral membrane protein localised in the podocyte [1,2]. Autosomal dominant FSGS may be due to an abnormality of alpha-actinin 4 or has a gene locus at 11q21-q22 [3,4]. In Frasier syndrome, caused by mutations within the intron 9 donor splice site of the *WT-1* gene, FSGS also occurs [5,6].

The aim of this study was to investigate the possibility that cases of non-familial FSGS are associated with *WT-1* splice site mutations or *NPHS2* gene mutations.

Methods

Patients

Ten patients with a proven histologic diagnosis of FSGS were included in this study. The relevant clinical data are summarised in table 1. All patients had normal genitalia and no dysmorphic features. In all patients the proteinuria did not disappear after the initial treatment by prednisone 60 mg/m^2/day for 6 weeks. Subsequent treatment with cyclosporin A and a low dose of prednisone, in one patient combined with mycophenolate mofetil, resulted in complete remission in two and a partial improvement in 4 patients.

Two patients (numbers 8 and 9) reached end-stage renal failure at the age of 8 and 14.5 years respectively.

More detailed information on patients 9 and 10 is provided, as DNA abnormalities were observed in both.

Table 1 Clinical data of the patients with idiopathic non-familial FSGS

Patient	Sex	Age at onset months	Data at admission			Immunosuppressive treatment	Response to treatment
			proteinuria g/l	serum albumin g/l	serum creatinine µmol/l		
1	M	12	19.2	20	35	csA, Pred	CR
2	M	10	37.9	18	54	csA, Pred	CR
3	F	128	11.1	18	51	csA, Pred	PR
4	M	138	15.9	15	54	csA, Pred, MMF	PR
5	F	22	49.4	19	141	csA, Pred	PR
6	M	160	2.2	32	119	Pred	PR
7	M	90	7.7	19	67	csA, Pred	PR
8	F	49	6.3	30	36	csA, Pred	no
9	F	103	14.5	23	51	csA, Pred	no
10	M	42	6.0	26	79	csA,Pred	no

M = male, F = female, csA = cyclosporin A, Pred = prednisone, MMF = mycophenolate mofetil, CR = complete remission and PR = partial remission.

Case 1

Patient 9, a white female, presented at the age of 8.5 years with general malaise, weight loss and abdominal pain. Her parents and two siblings were healthy. Physical examination was unremarkable; there was no peripheral oedema. Blood pressure was 120/80 mm Hg. Urinalysis revealed 14.5 g/l protein and 25-50 erythrocytes per high-powered field. Serum creatinine was 51 μmol/l, albumin - 23 g/l and cholesterol - 8.6 mmol/l. Complement factors C3 and C4 were normal. Hepatitis B, C and HIV serology was negative. Renal ultrasound was normal. She did not respond to the initial treatment with prednisone 60 mg/m^2/day during 6 weeks. Afterwards renal biopsy was performed. Light microscopic examination of renal tissue revealed FSGS in 4 glomeruli. The other 10 glomeruli showed slight mesangial hypercellularity. Patient 9 was treated with cyclosporin A and low dose prednisone with an initially partial response: decrease of proteinuria to 1-2 g/l and increase of serum albumin to 34-37 g/l. The treatment with enalapril had to be discontinued because of the development of urticaria. Four years later she developed an overt nephrotic syndrome despite the continuation of cyclosporin A treatment and the addition of prednisone and angiotensin II receptor antagonist. Renal function deteriorated gradually with the development of end stage renal disease at the age of 14.5 years. After the initiation of hemodialysis bilateral nephrectomy was performed because of the persistent nephrotic syndrome and severe hypertension. Histologic examination of both kidneys revealed no Wilms tumours. She is actually on the waiting list for renal transplantation.

Case 2

Patient 10, a white male, the only child of two healthy parents, presented at the age of 3.5 years with an upper airway infection. Physical examination revealed severe peripheral oedema and ascites. Blood pressure was 120/83 mm Hg. Urinalysis showed proteinuria 6 g/l and microscopic hematuria. Serum creatinine was 79 μmol/l, albumin - 26 g/l and cholesterol -10.8 mmol/l. Complement factors C3 and C4 were normal. As six weeks of prednisone treatment 60 mg/m^2/day gave no improvement, renal biopsy was performed. Microscopic examination of renal tissue revealed FSGS. Patient 10 was treated with cyclosporin A, low dose of prednisone and enalapril. After 18 months of treatment the nephrotic syndrome persisted and the renal function deteriorated.

DNA sequencing

Genomic DNA was isolated from whole blood using the salting-out method [7]. Amplification of 10 *WT-1* exons and 8 *NPHS2* exons was performed by PCR. The primers for the *WT-1* gene were developed in intronic sequences flanking the respective exons and are available on request (GenBank accession number AH003034). The primers used for the *NPHS2* gene (GenBank accession number AJ279246-AJ279253) have already been described by Boute *et al* [1]. The PCR products were analysed by DNA sequencing (Dye Terminator Cycle Sequencing, PE Applied Biosystems, Foster City, CA). The genomic DNA from 50 healthy controls was used to confirm mutations.

Results

WT-1 mutation analysis

After sequencing the total *WT-1* gene, we found one heterozygous *de novo* mutation in patient 9 (case 1). The mutation was not observed in the parents of patient 9. It concerns a mutation in intron 9, 1228+ 5 bp G>A substitution (figure 1a). Repeated PCR and DNA sequencing analysis affirmed this mutation in patient 9.

NPHS2 mutation analysis

In the *NPHS2* gene we found a heterozygous polymorphism in patient 5 and patient 7 in exon 5, 686 G>A (Arg229Gln). This polymorphism was found in three out of the hundred control alleles.

Beside this polymorphism we also found a heterozygous mutation in patient 10 (case 2), also in exon 5, 622 G>A (Ala208Thr) (figure 1b). This mutation was not found in hundred control alleles. The mutated allele turned out to be maternal, as we also detected the heterozygous mutation in the genomic DNA of the mother.

Repeated PCR and DNA sequencing analyses affirmed the polymorphism and mutation in the patients.

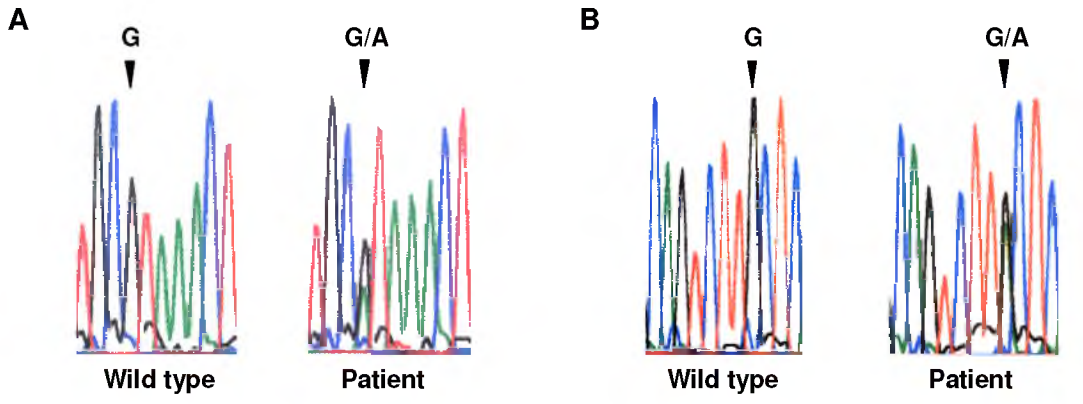

Figure 1 DNA sequencing results of patients 9 and 10. **A.** The heterozygous guanine (G) to adenine (A) substitution at position intron 9 1228 + 5 bp of the *WT-1* gene in patient 9.
B. The heterozygous guanine (G) to adenine (A) substitution at position 622 of the *NPHS2* gene, resulting in a heterozygous substitution of the amino acid Alanine to Threonine in patient 10. The patients are shown on the right, wild types on the left.

Discussion

Although a small group of patients was studied, two mutations were detected. In patient 9 an intron 9 splice site mutation within the *WT-1* gene was observed. It turned out to be a *de novo* mutation as it was not found in both parents. This mutation has been previously described in patients with Frasier syndrome, defined by male pseudohermaphroditisme, progressive glomerulopathy and frequent development of gonadoblastoma [8-10]. We found this mutation in a patient with normal female phenotype and XX karyotype. The same mutation has been found in a female, presenting with proteinuria at the age of 6 and developing an overt nephrotic syndrome during the pregnancy at the age of 27. She transmitted the mutation to her child, presenting with male pseudohermaphroditism and diffuse mesangial sclerosis [11]. Further study of 37 children with primary steroid-resistant FSGS revealed intron 9 mutations only in a patient with diaphragmatic hernia proximal hypospadias and unilateral testicular ectopia [12]. Our finding of a *WT-1* gene splice site mutation in 1 of 10 examined patients has practical implications for the patient as regular control of gonads and kidneys is required because of the risk of gonadoblastoma and rarely Wilms tumours.

In patient 10, one heterozygous mutation inherited from the mother was found in exon 5 of the *NPHS2* gene. Beside two families with compound heterozygous mutations, Boute *et al* also found only one heterozygous mutation in four families out of fourteen families [1]. Concerning the fact that *NPHS2* is responsible for the autosomal recessive form of steroid-resistant FSGS it is most likely these patients have a second mutation in the DNA sequence regulating the RNA splicing process or, alternatively, in the promoter region of the *NPHS2* gene.

During the review process of the present communication, Caridi *et al* reported in a group of 44 patients with steroid-resistant non-familial FSGS mutations in the *NPHS2* gene in 9 patients. Six patients had homozygous mutations and three patients had compound heterozygous mutations in the *NPHS2* gene [13]. This data sustain our results and suggest that mutational analysis of the *NPHS2* gene is a valuable approach in patients with idiopathic FSGS.

The finding of a polymorphism in exon 5 of the *NPHS2* gene in 2 of 10 examined patients versus 3 of 50 tested controls might rise a question concerning the role of *NPHS2* polymorphisms in the pathogenesis of focal segmental glomerulosclerosis.

Even in this small series the genetic diagnosis was important because the disorder will not recur after transplantation (NPHS2 mutations). Our observations support the necessity of testing for mutations in the *WT-1* and *NPHS2* gene in steroid-resistant non-familial FSGS, which shall be established in additional studies.

References

1. Boute N, Gribouval O, Roselli S *et al.* NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet.* 2000; **24:** 349-354.

2. Roselli S, Gribouval O, Boute N *et al.* Podocin localizes in the kidney to the slit diaphragm area. *Am J Pathol.* 2002; **160:** 131-139.

3. Winn MP, Conlon PJ, Lynn KL *et al.* Linkage of a gene causing familial focal segmental glomerulosclerosis to chromosome 11 and further evidence of genetic heterogeneity. *Genomics.* 1999; **58:** 113-120.

4. Kaplan JM, Kim SH, North KN *et al.* Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet.* 2000; **24:** 251-256.

5. Frasier SD, Bashore RA, Mosier HD. Gonadoblastoma associated with pure gonodal dysgenesis in monozygous twins. *J Pediatr.* 1964; **64:** 740-745.

6. Barbaux S, Niaudet P, Gubler MC *et al.* Donor splice-site mutations in WT1 are responsible for Frasier syndrome. *Nat Genet.* 1997; **17:** 467-70.

7. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988; **16:** 1215.

8. Bruening W, Bardeesy N, Silverman BL *et al.* Germline intronic and exonic mutations in the Wilms' tumour gene (WT1) affecting urogenital development. *Nat Genet.* 1992; **1:** 144-148.

9. Poulat F, Morin D, Konig A *et al.* Distinct molecular origins for Denys-Drash and Frasier syndromes. *Hum Genet.* 1993; **91:** 285-6.

10. Bardeesy N, Zabel B, Schmitt K, Pelletier J. WT1 mutations associated with incomplete Denys-Drash syndrome define a domain predicted to behave in a dominant-negative fashion. *Genomics.* 1994; **21:** 663-664.

11. Denamur E, Bocquet N, Mougenot B *et al.* Mother-to-child transmitted WT1 splice-site mutation is responsible for distinct glomerular diseases. *J Am Soc Nephrol.* 1999; **10:** 2219-2223.

12. Denamur E, Bocquet N, Baudouin V *et al.* WT1 splice-site mutations are rarely associated with primary steroid-resistant focal and segmental glomerulosclerosis. *Kidney Int.* 2000; **57:** 1868-1872.

13. Caridi G, Bertelli R, Carrea A *et al.* Prevalence, genetics, and clinical features of patients carrying podocin mutations in steroid-resistant nonfamilial focal segmental glomerulosclerosis. *J Am Soc Nephrol.* 2001; **12:** 2742-2746.

Chapter 3

Mitochondrial tRNA$^{Leu(UUR)}$ mutation in a patient with steroid-resistant nephrotic syndrome and focal segmental glomerulosclerosis

The heterogeneity of mitochondrial cytopathies is characteristic for this group of disorders, which preferentially affect the muscle and nerve system. The A3243G transition in the tRNA $^{LEU(UUR)}$ gene has been associated with slowly progressive forms of focal segmental glomerulosclerosis (FSGS). Here we present a patient who developed a severe nephrotic syndrome during her first pregnancy, which persisted after delivery, and proved resistant to immunosuppressive therapy. A sister of our patient had developed diabetes mellitus. We analyzed the DNA for the presence of the mtDNA A3243G transition. The DNA was isolated from peripheral blood leukocytes and urine sediments. Polymerase chain reaction was performed to amplify the mtDNA. Restriction enzyme analysis was used to detect the presence of the A3243G transition. Quantitative analysis of the A3243G mutation was done using the pyrosequencing technique and revealed a proportion of mutated mtDNA of 30% in the leukocytes and 68% in the urine sediments of the proband. On further analysis we also found the transition in the mother, the diabetic sister and the daughter of the proband. MtDNA abnormalities can cause a steroid-resistant nephrotic syndrome, histologically characterized by FSGS. Physicians should be especially mindful of mitochondrial abnormalities when hearing loss, diabetes mellitus, or neuromuscular disorders are present in the patient or family members.

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Introduction

Focal Segmental Glomerulosclerosis (FSGS) is a common cause of the nephrotic syndrome in adults. FSGS is a description of a histological lesion and not a disease entity. In recent years much progress has been made in unraveling the pathogenesis of (various forms) of focal glomerulosclerosis. It has been demonstrated that mutations in podocytic proteins such as podocin and α-actinin 4 are responsible for autosomal-recessive and autosomal-dominant forms of focal glomerulosclerosis [1]. Mutations in mitochondrial DNA (mtDNA) have also been associated with focal glomerulosclerosis.

Although mitochondrial cytopathies preferentially affect the muscle and nervous system, this group of disorders is characterized by their phenotypic heterogeneity. This large variety in clinical symptoms is due mainly to the co-existence of wild type and mutated mitochondrial DNA (heteroplasmy), unequally distributed between cells and organs [2].

A typical example of a mitochondrial cytopathy and its variety of symptoms is the A3243G transition in the $tRNA^{Leu(UUR)}$ gene. *In vitro* studies have shown that the A3243G transition is responsible for a mitochondrial respiratory chain defect and that it disturbs the protein synthesis in the mitochondrion [3].

This mtDNA mutation is associated mainly with the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) [4]. Occasionally, renal tubular dysfunction and FSGS have been associated with MELAS [5]. Several other clinical presentations associated with the A3243G mutation include chronic progressive external ophthalmoplegia (CPEO), diabetes mellitus, (cardio)myopathy, hearing loss and dystonia [6,7].

More recently, this mutation was associated with FSGS in patients who were not diagnosed as having a MELAS phenotype [8-15]. In most patients the clinical picture was characterized by the absence of a nephrotic syndrome and a slowly progressive renal failure.

We present a patient with a full blown, steroid-resistant nephrotic syndrome, histologically characterized by FSGS, with evidence of abnormal mitochondria in the podocyte and the detection of mutated mtDNA in urine and blood.

Methods

Molecular Genetic Study

DNA was isolated from peripheral blood leukocytes using the salting-out method. Also, urine sediments were used to isolate DNA. After centrifugation of the urine for 10 minutes at 3000 r.p.m., the pellet was washed with phosphate-buffered saline. DNA was extracted using a commercially available DNA isolation kit (Puregene™ DNA isolation kit; Gentra systems, MN).

Screening for the A3243G mutation was performed by polymerase chain reaction (PCR) using the following primers: 5'-CAACTTAGTATTATACCCACAC-3' and

5'-ATTAGAATGGGTACAATGAGGA-3'. The PCR conditions were 92°C for 30 s, 55°C for 30 s and 72°C for 60 s, for a total of 35 cycles leading to a PCR product of 162 bp. PCR products subsequently were digested overnight with *ApaI* restriction enzyme. The presence of the A3243G mutation results in an additional restriction site on position 104 bp of the PCR product. The restriction fragments were analyzed on a 1% agarose gel containing ethidium bromide to visualize the DNA.

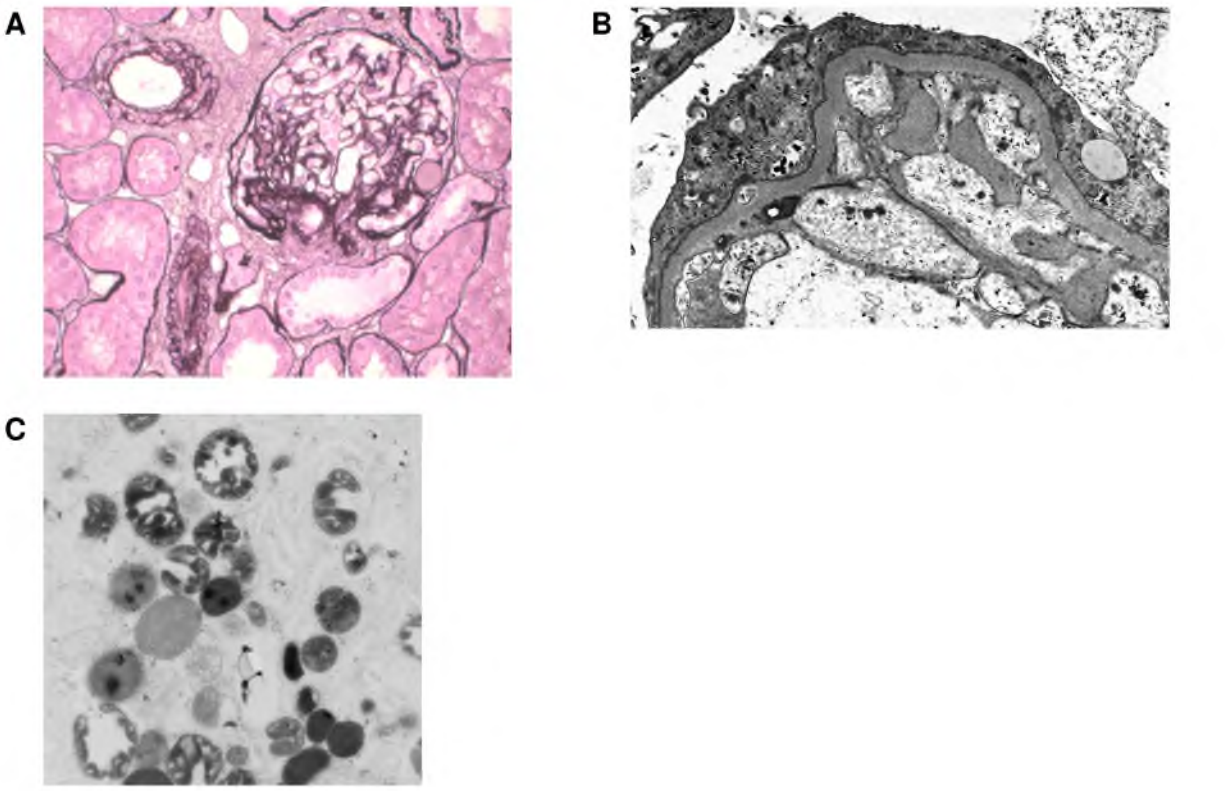

Figure 1 Light and electron microscopic pictures of the renal biopsy taken from the proband. **A.** Light microscopy, silver staining. Shown is a glomerulus with peri-hilar segmental sclerosis, hyalinosis and an adhesion, consistent with an FSGS lesion. Arterioles and small arteries show moderate hyalinosis, subendothelially and in the media with a spot-like distribution, consistent with loss of smooth muscle fibers. **B.** Electron microscopy, original magnification 3000X. Glomerular segment with extensive foot process effacement of the podocytes. Most podocytes showed condensation of the actin cytoskeleton and mild vacuolation of the cytoplasm. Multinucleated podocytes were not encountered. The endothelium was swollen and vacuolated. The glomerular basement membrane has normal structure and width. Electron-dense deposits were observed in the paramesangial regions (not shown). **C.** Electron microscopy, original magnification 10 000x. Mitochondria in podocytes show abnormal morphology. Cristae appear irregular and frequently a large central vacuole with irregular outline is present. There appears to be transformation of mitochondria into lysosome-like vesicles, suggesting degradation of mitochondria.

Positive DNA samples were analyzed quantitatively using Pyrosequencing™ technology (Pyrosequencing, Upsala, Sweden). Pyrosequencing was performed according to the protocol of the manufacturer. PCR of a mtDNA fragment containing the 3243 position was amplified using the following primers: universal primer (biotinylated), 5'-GGGACACCGCTGATCGTTTA-3'; forward primer 5'-GACGGGACACCGCTGATCGTTTACAACTTAGTATTATACCCACAC-3'; and reverse primer 5'- ATTAGAATGGGTACAATGAGGA-3'. PCR was carried out in a 50 µl volume containing 0.02 µM forward primer, 0.2 µM reverse primer and 0.2 µM of the biotinylated universal primer. PCR conditions were 92°C for 30 s, 55°C for 30 s, 72°C for 30 s, for a total of 40 cycles. Single-stranded template DNA, which in the present assay is the forward strand of the fragment, was purified using streptavidin-coated Sepharose beads. The actual pyrosequencing was performed on the PSQ96 platform using sequence primer 5'-TATGCGATTACCGGGC-3'.

In a pyrosequence reaction, the four different deoxynucleotide triphosphates (dNTPs) are added separately one after the other. The incorporation of dNTP is accompanied by release of pyrophosphate (PPi). This PPi is involved in a light-producing reaction of which the amount of light produced is proportional to the number of nucleotides incorporated (figure 3C). The light is detected by a charge coupled device camera and seen as a peak in a pyrogram. Apyrase, a nucleotide-degrading enzyme, continuously degrades ATP involved in the light-producing reaction, and unincorporates dNTPs. This switches off light production and regenerates the reaction solution. Because the forward strand is used as template in the sequencing reaction, the change detected in the present assay concerns a T to C exchange. In fact, the amount of dTTP and dCTP incorporated at position 3243 during the sequencing reaction was determined in this way and from this the percentage of heteroplasmy was calculated.

Results

Clinical history of the proband

Our patient was referred to our out-patient clinic at the age of 31 years because of a nephrotic syndrome that persisted after pregnancy. Dipstick-positive proteinuria was noted in the first trimester of pregnancy. At the 27th week, she developed pre-eclampsia and a Caesarean section was performed because of fetal distress. Six weeks after pregnancy termination, she visited the out-patient clinic.

The medication consisted of enalapril 5 mg twice daily, and bumetanide 1 mg twice daily. Her medical history was unremarkable. The family history was negative for renal diseases, deafness, or diabetes. At physical examination blood pressure was 132/80 mmHg. There was moderate oedema. Abdominal ultrasound revealed two normal sized kidneys. Laboratory investigation revealed a nephrotic syndrome and normal renal function: serum creatinine 73 µmol/l, serum albumin 26 g/l and serum cholesterol 11.3 mmol/l. Proteinuria ranged from 8.2 to 15.8 g/10 mmol creatinine, and urinary β_2-microglobulin was 400 µg/24h

(normal value ≤ 240 µg/24h). The selective index calculated as clearance of IgG/clearance according to the Cockcroft and Gault formula was 101 ml/min. The urinary sediment revealed only sporadic erythrocytes, and many oval fat bodies.

Because of the persistence of the nephrotic syndrome, a renal biopsy was performed, ~6 months after pregnancy. On light microscopy, characteristic lesions of focal glomerulosclerosis were present in five of eight glomeruli (figure 1a). There was no evidence of mesangial cell proliferation. We observed focal tubulo-interstitial infiltrate and tubular atrophy. Arterioles and small arteries showed moderate hyalinosis, both subendothelially and in the media, in a spot-like distribution, consistent with loss of smooth muscle cells. On immunofluorescence, we observed focal segmental coarse granular deposits of IgM and C3 consistent with FSGS. In addition, there were granular depositions of IgA in the mesangium and to a lesser extent in the capillary walls. Electron microscopy showed diffuse podocytic foot process effacement (figure 1b). The endothelium was often swollen and vacuolated. Paramesangial deposits were noted. In view of the clinical presentation and histological picture, we favoured a diagnosis of idiopathic focal glomerulosclerosis occurring in a patient with IgA deposits of undetermined significance (see Discussion).

Treatment consisted of enalapril 20 mg, losartan 25 mg, bumetanide 1-2 mg and atorvastatine 20 mg. Blood pressure was relatively well regulated, with values varying between 138/78 and 156/84 mmHg. Since proteinuria persisted at levels above 8 g per day, and renal function deteriorated with a rise in serum creatinine to levels of 112 µmol/l, immunosuppressive therapy was instituted. However, successive treatment with prednisone, cyclophosphamide and finally plasmapheresis were all unsuccessful (figure 2).

Steroid treatment was complicated by the development of diabetes mellitus, which required oral antidiabetic therapy. At that time, patient informed us that her sister had in the meantime developed insulin-dependent diabetes mellitus. Because of our interest in the genetics of steroid-resistant focal glomerulosclerosis, we performed analysis for mtDNA mutations in DNA recovered from the blood and the urine.

Because of the abnormality found in the mtDNA, podocytes were visualized at greater magnification by electron microscopy. The pictures revealed abnormal mitochondria in the visceral epithelial cells (figure 1c). At present, patient is seen regularly at the out-patient clinic, with moderate proteinuria and slowly progressive renal failure (figure 2). In view of the observed mitochondrial mutation (see below), the patient was referred to the oto-rhino-laryngologist who found a hearing loss of 35 dB. Echocardiography disclosed concentric left ventricular hypertrophy, with a left ventricular mass index of 140 g/m^2 (normal <110 g/m^2). There was no evidence of asymmetrical hypertrophy of the interventricular septum.

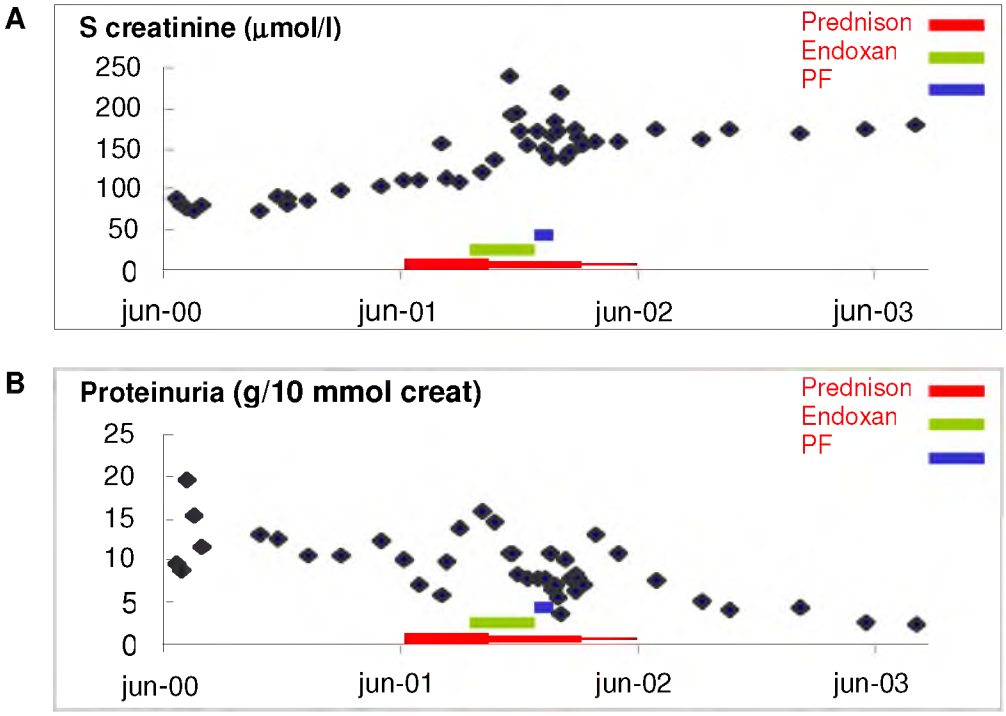

Figure 2 Time course of serum creatinine (A) and proteinuria (B) in the proband.
Because of a rise in serum creatinine and persistent proteinuria > 8g/day, treatment was instituted. Successive treatment with prednisone, cyclophosphamide and plasmapheresis (PF) as indicated was unsuccessful.

Molecular genetic study

We isolated DNA from peripheral blood leukocytes and urine sediment and tested the DNA for the presence of the A3243G transition in the tRNA $^{LEU(UUR)}$ gene. We found the mutation in both DNA samples after digesting the PCR fragment with *ApaI*. Quantitative analysis revealed a proportion of mutated mtDNA of 30% in the leukocytes and 68% in the urine sediments.

We also tested DNA of the maternal relatives of the patient. The results are shown in figure 3. The mother of the proband revealed no mutation in her leukocytes, but a positive proportion of 62% in the urine sediments. To our knowledge, the mother has no clinical symptoms related to the A3243G mutation. The sister of the proband, who developed insulin dependent diabetes mellitus, was also found positive for both DNA samples. A proportion of 25% was found in the leukocytes and of 28% in her urine sediments. Finally the daughter of the proband, who has normal renal function and no proteinuria, revealed the mtDNA mutation in a proportion of 51% in her leukocytes and 52% in her urine sediments.

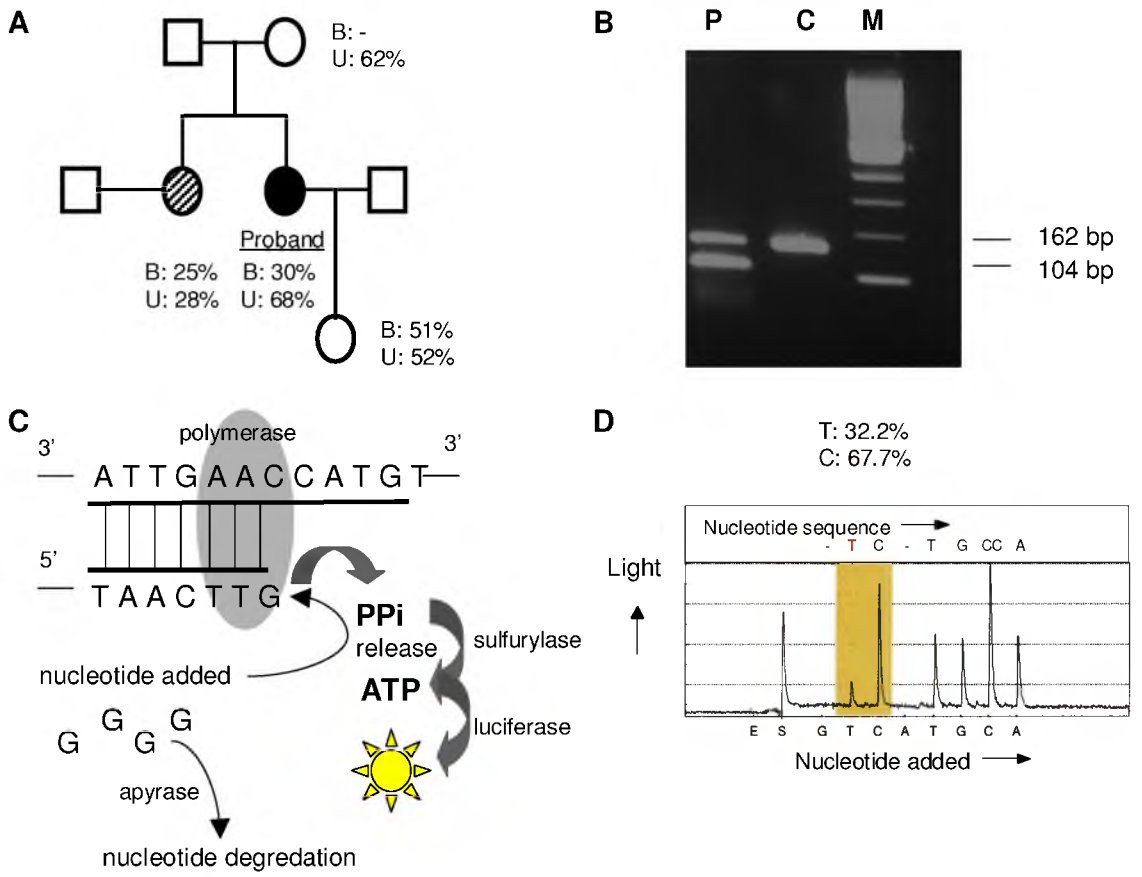

Figure 3 Family tree and results from the mtDNA A3243G transition analyses.
A. Family tree of the proband. The proband with a nephrotic syndrome is shown in black. The striped circle represents her sister who was diagnosed with diabetes mellitus. The percentages indicate the proportion of the A3243G transition in the tRNA$^{Leu(UUR)}$ gene found in the different DNA samples. B = peripheral blood leukocytes; U = urine sediments. **B.** PCR fragments from the proband (P) and a normal control subject (C) after digestion with *Apa*I. The 162-bp PCR fragment is digested to 104 and 58 bp products when the transition is present. M = marker. **C.** The principle of pyrosequencing, which is described in Methods. **D.** Pyrogram of the proband obtained from DNA isolated from urine sediment. The percentage T indicates the amount of thymine and C the amount of cysteine to which thymine is mutated. Before addition of the nucleotides, enzymes (E) and substrate (S) are added.

Discussion

To our knowledge, our case report is the first to document the development of FSGS and a severe steroid-resistant nephrotic syndrome in an adult patient with the mitochondrial A3245G transition. Mutated mtDNA amounted 30% in leukocytes and 68% in cells derived from urine sediments. Abnormal mitochondria were present in the podocytes. The mutation was also found in the family members, notably in a sister with diabetes mellitus.

Admittedly, we must consider whether this patient had IgA nephropathy in view of the deposits observed by immunofluorescence and electron microscopy. However, we feel that in our patient the clinical presentation and histological data favour a diagnosis of primary FSGS co-occurring with the incidental finding of IgA deposits. Specifically, we did not observe mesangial proliferative lesions; the patient never had episodes of macroscopic or microscopic haematuria. it is well known that IgA deposits in ++ to +++ intensity can be found incidentally in biopsies of otherwise healthy cadaveric donors (reported incidence 7-9%), and even in living donors without urine abnormalities. Furthermore, many patients have been reported who presented with a glomerular disease and displayed mesangial IgA deposits, the most characteristic being patients with minimal change disease [16]. In a recent study, 18 patients with FSGS and IgA deposits were described, clearly demonstrating that these patients presented with a clinical picture and a prognosis that closely resembled that of patients with typical primary FSGS [17]

The interest of nephrologists for mitochondrial diseases has been fostered by publications concerning the involvement of the kidney in mitochondrial cytopathies [5,8]. tRNA mutations affects multiple pathways and can be traced to the destabilization of structural features that destroy the native tRNA conformation required for protein synthesis efficiency, aminoacylation, post-transcriptional modification and processing. The A3243G mutation alters the A14 nucleotide that is highly conserved in the $tRNA^{Leu}$. The mtDNA A3245G transition has been associated with the MELAS syndrome, and more recently with maternally inherited diabetes mellitus, hearing loss, cardiomyopathy and chronic progressive external ophtalmoplegia [6,7]. It has become evident that the mutation can also cause isolated renal disease in patients without signs of MELAS even after prolonged follow-up [8-15].

In case reports and small studies, >30 patients have been documented with the mtDNA A3245G transition and FSGS [8-15]. Clinically, most patients had non-nephrotic proteinuria (<3g/day) and a progressive deterioration of renal function. In only two adult patients was proteinuria in the nephrotic range. One male patient was diagnosed at the age of 18 years with diabetes mellitus, which was very poorly controlled in the following years [13]. At the age of 23 years, he had developed a severe renal insufficiency (serum creatinine 522 µmol/l) and proteinuria of 3.5 g/day. Renal biopsy disclosed numerous globally sclerotic glomeruli, an increase of mesangial matrix, thickened glomerular basement membrane on electron microscopy, and no foot process fusion. Also, abnormal mitochondria were not found in the podocytes. These data strongly suggest that diabetic nephropathy was a major cause of the renal disease. The second patient was a 50-year-old female, with a 4 year

history of diabetes, who was referred with severe renal failure (creatinine clearance 15 ml/min), polycystic kidneys and a proteinuria of 3g/day, below the nephrotic range [12].

Although our patient was not aware of any hearing loss, an abnormal audiogram was noted. Hearing loss was also frequently noted in the described patients, and, in most, hearing loss preceded the development or discovery of proteinuria [11,12,14]. Since hearing loss was often familial, it is not a surprise that many patients had been diagnosed with Alport's syndrome [8,9]. Diabetes mellitus was also a frequent finding; however, in most patients, diabetes mellitus became manifest only during follow-up, after the discovery of proteinuria or renal disease [10,12,15].

Information on renal biopsy findings is somewhat limited. In the available biopsies there was evidence of FSGS with various percentages of globally sclerotic glomeruli [9-14]. In isolated cases, hyalinosis of the arterioles with evidence of smooth muscle cell necrosis and the presence of multinucleated podocytes has been reported [10,15]. Most characteristic was the finding of abnormal mitochondria in podocytes and tubular cells, reported in eight out of ten patients studied by electron microscopy [9-13].

Our patient disclosed the typical lesions of FSGS, and abnormal mitochondria were observed in the podocytes, although we must admit that these abnormalities were identified only when we specifically looked at higher magnification after discovery of the mtDNA mutation. We did not observe multinucleated podocytes. Notably, as described by others, we also observed some abnormalities of the vessel wall, with hyalinosis in a spot-like pattern, consistent with loss of smooth muscle cells [10-15]. These findings of vessel wall abnormalities have not been not emphasized thus far; however, they might suggest that dysfunction of the mitochondria in the vascular endothelial or smooth muscle cells could result in early vascular damage.

We analysed the presence of the A3243G transition in blood leukocytes and urine sediments. Patients harboring this mutation usually have higher percentages of mutated mtDNA in muscle or urine sediments than in cells derived from rapidly dividing tissues such as bone marrow. Since urine sediments contain podocytes but also tubular epithelial cells and cells of the lower urinary tract, the measured heteroplasmic load does not represent the actual load in the podocytes.

Chinnery *et al.* found no relationship between the level of A3243G in blood and the frequencies of any of the clinical features they assessed, such as recurrent stroke-like episodes, CPEO, diabetes mellitus, pigmentary retinopathy, deafness, dementia, ataxia and myopathy [7]. In contrast, they found a correlation between the levels found in muscle and certain clinical features, particularly if the amount of mutated mtDNA exceeded the 15% level. For example, the observed frequency of recurrent strokes rose from zero in individuals with 21-60% A3243G in muscle to 94% for individuals with 91-100% in muscle.

Although the literature data are limited, it is notable that the majority of patients with renal diseases and an A3243G mutation are female. Furthermore, in the female patients, the disease was often more severe. This suggests that the phenotypic (renal) expression of this mitochondrial cytopathy is influenced by hormonal factors. However, the preponderance of females is as yet unexplained.

Should we screen all patients with primary FSGS for mitochondrial mutations? At present, the evidence in favour of such a strategy is lacking. In fact, two Japanese studies did not find the A3245G transition in 25 and 17 patients with primary non-familial FSGS respectively [10,11]. Clinicians should be suspicious of patients with steroid-resistant FSGS who present with some particular features such as deafness, diabetes, neuromuscular symptoms, cardiomyopathy or a family history positive for any of these. In conclusion, the mtDNA A3245G transition can be associated with FSGS and severe nephrotic syndrome. Clinicians should be aware of the clinical heterogeneity of mitochondrial cytopathies, which will allow earlier detection of these disorders. It is advised that patients with mtDNA mutations should be followed closely to detect the development of associated conditions such as diabetes mellitus and cardiomyopathy.

References

1. Pollak MR. The genetic basis of FSGS and steroid-resistant nephrosis. *Semin Nephrol.* 2003; **23:** 141-146.

2. Schmiedel J, Jackson S, Schafer J, Reichmann H. Mitochondrial cytopathies. *J Neurol.* 2003; **250:** 267-277.

3. King MP, Koga Y, Davidson M, Schon EA. Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the tRNA(Leu(UUR)) mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes. *Mol Cell Biol.* 1992; **12:** 480-490.

4. Piechota J, Mroczek K, Bartnik E. MELAS as an example of a mitochondrial disease. *J Appl Genet.* 2001; **42:** 351-358.

5. Mochizuki H, Joh K, Kawame H, Imadachi A *et al.* Mitochondrial encephalomyopathies preceded by de-Toni-Debre-Fanconi syndrome or focal segmental glomerulosclerosis. *Clin Nephrol.* 1996; **46:** 347-352.

6. Sudarsky L, Plotkin GM, Logigian EL, Johns DR. Dystonia as a presenting feature of the 3243 mitochondrial DNA mutation. *Mov Disord.* 1999; *14:* 488-491.

7. Chinnery PF, Howell N, Lightowlers RN, Turnbull DM. Molecular pathology of MELAS and MERRF. The relationship between mutation load and clinical phenotypes. *Brain.* 1997; **120:** 1713-1721.

8. Jansen JJ, Maassen JA, van der Woude FJ *et al.* Mutation in mitochondrial tRNA(Leu(UUR)) gene associated with progressive kidney disease. *J Am Soc Nephrol.* 1997; **8:** 1118-1124.

9. Cheong HI, Chae JH, Kim JS *et al.* Hereditary glomerulopathy associated with a mitochondrial tRNA(Leu) gene mutation. *Pediatr Nephrol.* 1999; **13:** 477-480.

10. Hotta O, Inoue CN, Miyabayashi S *et al.* Clinical and pathologic features of focal segmental glomerulosclerosis with mitochondrial tRNALeu(UUR) gene mutation. *Kidney Int.* 2001; **59:** 1236-1243.

11. Yamagata K, Muro K, Usui J *et al.* Mitochondrial DNA mutations in focal segmental glomerulosclerosis lesions. *J Am Soc Nephrol.* 2002; **13:** 1816-1823.

12. Guery B, Choukroun G, Noel LH *et al.* The spectrum of systemic involvement in adults presenting with renal lesion and mitochondrial tRNA(Leu) gene mutation. *J Am Soc Nephrol.* 2003; **14:** 2099-2108.

13. Nakamura S, Yoshinari M, Doi Y *et al.* Renal complications in patients with diabetes mellitus associated with an A to G mutation of mitochondrial DNA at the 3243 position of leucine tRNA. *Diabetes Res Clin Pract.* 1999; **44:** 183-189.

14. Kurogouchi F, Oguchi T, Mawatari E *et al.* A case of mitochondrial cytopathy with a typical point mutation for MELAS, presenting with severe focal-segmental glomerulosclerosis as main clinical manifestation. *Am J Nephrol.* 1998; **18:** 551-556.

15. Doleris LM, Hill GS, Chedin P *et al.* Focal segmental glomerulosclerosis associated with mitochondrial cytopathy. *Kidney Int.* 2000; **58:** 1851-1858.

16. Association of IgA nephropathy with steroid-responsive nephrotic syndrome. A report of the Southwest Pediatric Nephrology Study Group. *Am J Kidney Dis.* 1985; **5:** 157-164.

17. Haas M. IgA nephropathy histologically resembling focal-segmental glomerulosclerosis: a clinicopathologic study of 18 cases. *Am J Kidney Dis.* 1996; **28:** 365-371.

Chapter 4

A genetic childhood disease with consequences in adult life: the Denys-Drash syndrome

In a 17-year-old woman with absent sexual development and a congenital nephrotic syndrome leading to renal failure, the Denys-Drash syndrome was diagnosed after development of an ovarian dysgerminoma. The Denys-Drash syndrome is characterized by the triad: progressive nephropathy due to diffuse mesangial sclerosis, male pseudo-hermaphroditism (XY karyotype with ambiguous or female genital organs) and an increased risk of developing Wilms tumour and gonadoblastoma. The syndrome is generally caused by a genetic defect in the Wilms tumour suppressor 1 gene (*WT1* gene). A WT1 mutation and XY karyotype were also found in our patient. The *WT1* gene encodes a transcription factor playing an important role in renal and genital development. The diagnosis of Denys-Drash syndrome had important consequences for the treatment and follow-up of the patient. The second gonad and the native kidneys were removed due to the increased risk of malignancy. Moreover, the finding of a XY karyotype could result in serious psychological problems. Physicians responsible for the health of adults are confronted more and more often with the consequences of childhood diseases. This case illustrates the necessity to inform such physicians about previously untreatable genetic diseases of childhood in order to provide adequate medical management of these patients.

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A growing number of diseased children reach adult life due to progress in medical treatment. Physicians responsible for the treatment of adult patients are therefore more often confronted with, for them, unknown paediatric disorders and genetic defects of which the symptoms manifests itself not sooner than in adult life or are still present at adult age.

Here we describe a case illustrating the complex care arising when a genetic paediatric disorder is diagnosed in adult life.

Case Report

Patient A, a 17-year old female, was first seen at the out-patient clinic Medical Oncology because of a dysgerminoma in the right abdomen (figure 1). The serum concentration of α-foetoprotein was 3.9 µg/l (normal <10), human choriongonadotrophin (β-HCG) 540 ng/ml (normal <2), and the lactatedehydrogenase (LDH) activity was 1901 U/l (normal <450). The tumour capsule was damaged during surgical removal.

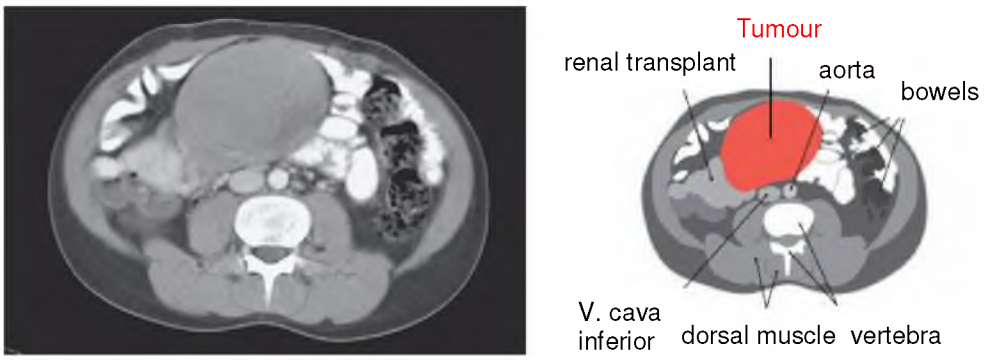

Figure 1 CT scan from patient A's abdomen showing a large dysgerminoma of the right ovary.

The medical history of the patient shows a congenital renal insufficiency due to diffuse mesangial sclerosis (DMS) with nephrotic syndrome (figure 2). She underwent renal transplantation at 4-, 5-, and 16-years of age. The first transplant was removed several months after transplantation because of untreatable acute rejection. The second transplant was lost as a result of chronic rejection. The function of the third transplanted kidney was stable with immunosuppressive treatment: prednisone, tacrolimus and mycophenolate, with an endogenous creatinine clearance of 80 ml/min/1.73m^2.

The patient was very small (body length of 153 cm) and completely pre-pubic. Examination using X-ray revealed a skeletal age of 12 years. Adjuvant chemotherapy with 2 cycles carboplatin-etoposide-bleomycin was started because of the dysgerminoma and the damaged capsule during surgery. Since this treatment is nephrotoxic, the carboplatin was replaced by cisplatin and the bleomycin was administered in a lower dose.

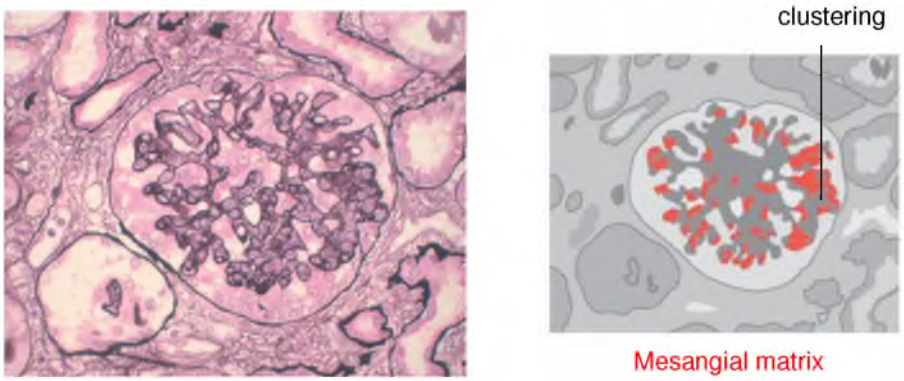

Figure 2 Histological image of the kidney with diffuse mesangial sclerosis in the Denys-Drash syndrome; in the centre a glomerulus is seen with striking collapses of the capillary tuft, epithelial hyperplasia, and expansion of the mesangial matrix which can be recognized by the increase of black deposits. (silverstaining, ~ 260x magnified)

The renal insufficiency due to DMS with nephrotic syndrome pointed in the direction of the Denys-Drash-syndrome (DDS). This syndrome is characterized by (a) renal insufficiency due to DMS; (b) male pseudo-hermaphroditism with gonadal dysgenesis, XY-karyotype with ambiguous or female genital organs; and (c) Wilms tumour and a higher risk of developing gonadoblastoma which can degenerate in malignant dysgerminoma. When a dysgerminoma is diagnosed it is advisable to remove the second gonad as well since there is a higher risk of malignant degeneration of the remaining gonad.

In patient A genetic analysis was advisable because of the possible therapeutic consequences; the removal of the second gonad and the rudimentary native kidneys due to the higher risk of malignancies. Before the analysis was performed, a plan was set up to guide the patient through psychological and socio-emotional problems which may arise when a XY-karyotype is confirmed. The parents were completely informed and when the patient turned out to have a XY-karyotype, they would gradually inform their daughter with the help of a professional team.

The genetic analysis indeed revealed a XY-karyotype and also a *de novo* mutation in the Wilms-tumoursuppressor-1 (*WT1*) gene (1025 T>G, M342R), which confirms the diagnosis 'Denys-Drash syndrome'. This mutation was described before and was found in a DDS patient with female genitalia and a 46XY-karyotype [1]. Our patient was informed and advised to remove the remaining gonad as well, considering the high risk of malignant degeneration. To induce puberty, hormonal substitution therapy was started consisting of ethinylestradiol, later also with progestatives. Since the risk of developing of a Wilms tumour in the patients rudimentary kidneys was substantial, we also advised her to remove them as well.

First the remaining gonad was removed by explorative laparoscopy. Pathological examination revealed a gonadoblastoma, a benign abnormality and possibly a pre-stage of for example a dysgerminoma. Gonadoblastoma were localised all over the peritoneum and since a complete removal could not be guaranteed, regular check-ups, especially the

determination of tumour markers, was advised. Both kidneys showed nephroblastosis, also a benign abnormality and possibly a pre-stage of a Wilms tumour.

The secondary gender features developed completely in time and with the cyclic administration of progestatives the patient menstruated. At the last follow-up, at the age of 19 years, she was fully informed about her XY-karyotype. We intentionally choose to inform the patient completely at the end of her sexual development and puberty. As yet the patient did not have many problems getting over her diagnosis.

Consideration

Nowadays patients survive a genetic and before untreatable condition much longer and this makes it necessary for physicians who are responsible for the treatment of adults to get notice of these conditions. Congenital (before the age of 1 month) or infantile (before the age of 1 year) nephrotic syndrome belongs to this category of conditions which in these days, due to helpful supporting treatments and the possibility to receive renal transplantation, have a better outcome.

Early nephrotic syndrome usually develops due to mutations in genes expressed in the glomerular podocytes. DMS, isolated or seen in DDS, is mostly caused by heterozygous mutations in the *WT1* gene (chromoxome 11p13). Mutations in this gene are also found in patients with the Frasier syndrome (FS). This syndrome strongly resembles DDS and is characterized by focal segmental glomerulosclerosis, usually after the age of 5 years, absent virilisation with a 46XY karyotype, and a higher risk of developing gonadoblastoma, and more rarely Wilms tumours [2].

The *WT1* gene consists of 10 exons (figure 3) coding a 52-54 kD transcriptionfactor. In most cases DDS patients carry missense mutations in exon 8 or 9 resulting in an amino acid substitution. Mutations in exon 6 and 7 are found less often. Most mutations are *de novo* mutations although familial cases have been reported [3]. FS patients usually have intron 9 mutations affecting the alternative splice site of exon 9 [4].

WT1 is essential for a normal development of gonads and kidneys. Postnatal WT1 is exclusively expressed in glomerular podocytes, Sertoli-cells of the testis, and granulosa cells of the ovaries [5].

Patient A with congenital nephrotic syndrome due to DMS was diagnosed with DDS only after developing a dysgerminoma of the ovaries. She was not diagnosed at an earlier age because of the complete absence of virilisation despite the 46XY karyotype. This is very uncommon in DDS patients and is more characteristic for FS [6]. These common characteristics even emphasize more the suggestion that FS and DDS are not single disease entities but the boundaries of a broad spectrum [7].

Consequences of DDS diagnosis

For our patient the DDS diagnosis had important consequences for further treatment and follow-up:

- Establishing a XY-karyotype in a girl and informing her about this finding may lead to psychological problems. It is therefore advisable to consult professionals, physicians and psychologists, with experience in these conditions. Often the family of the patient is informed first and a confidant is asked to inform the patient. Correct and complete information about the condition from the physician, written information and contact with other DDS patients are important to get over the emotional impact of this difficult diagnosis. An interest group was founded for girls with a XY-karyotype (www.aisnederland.nl).
- The removal of the second gonad because of a higher risk of malignant degeneration. Patients with a *WT1* mutation have a higher risk of developing gonadoblastoma and dysgerminoma. The exact percentage of DDS patients who develop these tumours is unknown. About 90% of the patients with gonadal dysgenesis develop gonadal neoplasms [8]. Gonadoblastoma are stromal neoplasms, non-metastasising in-situ germ-cell malignancies. Gonadoblastoma may degenerate to dysgerminoma. These are fast growing and metastasising tumours. The are no clear guide lines concerning gonadectomy in patients with a *WT1* mutation, a XX-karyotype, normal female genitalia and echographically normal ovaries. We advise to leave the gonads in situ and to perform echographical screenings for gonadoblastoma each year in these patient.
- The rudimentary kidneys were removed because of a higher risk of developing Wilms tumour. It is unclear whether or not the presence of undifferentiated kidney tissue found in the rudimentary kidneys of patient A meant she still had a risk in developing Wilms tumour. According to some, all DDS patients develop a Wilms tumour before the age of 5 years [9]. This was not the case in patient A, again reflecting the diversity of this syndrome. Since the risk of developing a Wilms tumour is high, especially in DDS patients, it is advisable to perform bilateral nephrectomy when patients reach terminal renal insufficiency. However, this is not advisable before the age of 1 year, since the renin-angiotensin system plays an important role in the regulation of the blood pressure in infants and renin is produced by the kidney [10].

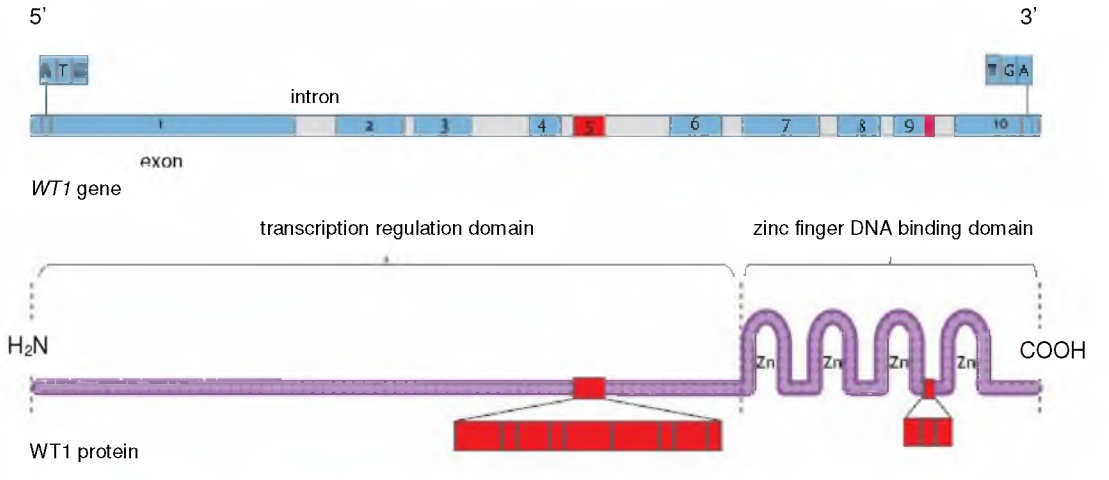

Figure 3 Schematic representation of the Wilms tumour suppressor 1 gene (upper part) and the WT1 protein: the 10 exons of the gene are depicted as numbered rectangles and the alternative splice sites in exon 5 and 9 are colored red and results in 17 or 3 extra amino acids respectively. The transcription regulating domain of the WT1 protein is encoded by exon 1-6; the zinc finger binding domain by exon 7-10.

Conclusion

Mutation analysis in the *WT1* gene and karyotyping should be performed in all patients with DMS, also in patients with focal segmental glomerulosclerosis and ambiguous genitalia.

When at the age of 14 years the patient has not reached puberty yet, also in patients with renal insufficiency, we recommend chromosomal analysis.

The physician should be careful in informing the diagnosis to the person involved considering the psychological and socio-emotional aspects of this syndrome.

References

1. Takata A, Kikuchi H, Fukuzawa R *et al.* Constitutional WT1 correlate with clinical features in children with progressive nephropathy. *J Med Genet.* 2000; **37:** 698-701.

2. Salomon R, Gubler MC, Niaudet P. Genetics of the nephrotic syndrome. *Curr Opin Pediatr.* 2000; **12:** 129-134.

3. Denamur E, Bocquet N, Mougenot B *et al.* Mother-to-child transmitted WT1 splice-site mutation is responsible for distinct glomerular diseases. *J Am Soc Nephrol.* 1999; **10:** 2219-2223.

4. Klamt B, Koziell A, Poulat F *et al.* Frasier syndrome is caused by defective alternative splicing of WT1 leading to an altered ratio of WT1 +/-KTS splice isoforms. *Hum Mol Genet.* 1998; **7:** 709-714.

5. Wagner KD, Wagner N, Schedl A. The complex life of WT1. *J Cell Sci.* 2003; **116:** 1653-1658.

6. Manivel JC, Sibley RK, Dehner LP. Complete and incomplete Drash syndrome: a clinicopathologic study of five cases of a dysontogenetic-neoplastic complex. *Hum Pathol.* 1987; **18:** 80-89.

7. Kohsaka T, Tagawa M, Takekoshi Y *et al.* Exon 9 mutations in the WT1 gene, without influencing KTS splice isoforms, are also responsible for Frasier syndrome. *Hum Mutat.* 1999; **14:** 466-470.

8. Slowikowska-Hilczer J, Romer TE, Kula K. Neoplastic potential of germ cells in relation to disturbances of gonadal organogenesis and changes in karyotype. *J Androl.* 2003; **24:** 270-278.

9. Habib R. Nephrotic syndrome in the 1st year of life. *Pediatr Nephrol.* 1993; **7:** 347-353.

10. van Lieburg AF, Monnens LA. Persistent arterial hypotension after bilateral nephrectomy in a 4-month-old infant. *Pediatr Nephrol.* 2001; **16:** 604-605.

Chapter 5

Focal Segmental Glomerulosclerosis in a patient homozygous for a CD2AP mutation

Focal segmental glomerulosclerosis (FSGS) is a histologic diagnosis in several kidney diseases characterized by proteinuria and a severe decrease in kidney function. Mutations in several genes were found in patients with primary FSGS, one of which is a CD2-associated protein, *CD2AP* (originally referred to CMS). This gene encodes an adaptor protein that plays a role in endocytosis, cell motility and cell survival. Mice deficient of Cd2ap (the mouse homolog) die due to kidney failure, while heterozygous mice develop lesions similar to those of FSGS patients. In the kidney, CD2AP regulates the actin cytoskeleton. The only previously described patient with *CD2AP* mutation had a severely truncated protein. In this study, we describe a patient with a novel mutation resulting in a premature stop codon yielding a protein truncated by only 4%. This shortened CD2AP protein displays a significantly decreased F-actin binding efficiency *in vitro* with no expression of the mutated allele in the patient's lymphocytes. Heterozygous expression of the CD2AP mutation in both parents did not lead to any kidney pathology, as both have normal glomerular filtration rates and no proteinuria.

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Introduction

Increasing numbers of patients, even in non-familial cases, are reported with focal segmental glomerulosclerosis (FSGS) owing to mutations in well-characterized genes. One of these genes is *CD2AP* (CD2-associated protein, originally named CMS).

Cd2ap, the mouse homolog of CD2AP, was first found in a yeast two-hybrid screen as a protein binding to the mouse T-cell membrane protein CD2, where it plays a role in clustering CD2 and polarizing the T-cell [1]. The role of Cd2ap in the kidney became apparent when Cd2ap knockout mice developed severe kidney problems and Cd2ap haploinsufficiency led to lesions resembling the human condition FSGS [2,3].

The human homolog CD2AP was found to directly bind $p130^{Cas}$, a docking protein, which appears to play a role in the integrin-mediated cell adhesion to the extracellular matrix [4]. Over the years, more and more proteins were found to interact directly with CD2AP/Cd2ap and its function in several cellular systems became apparent (figure 1 and table 1). The direct interaction of the COOH terminus of Cd2ap with F-actin suggests that Cd2ap is involved in the regulation of the actin cytoskeleton [15]. Indeed, several studies showed interaction of CD2AP/Cd2ap with proteins involved in cytoskeletal remodeling and cell motility [7-9,20]. More recently it became apparent that CD2AP/Cd2ap plays a role in endocytosis [10-14], in the transforming growth factor-β-induced apoptosis, and in the phosphatidylinositol 3-kinase/AKT survival pathway as well [19,21,22].

The CD2AP protein consists of 639 amino acids (aa) encoded by 18 exons (chromosome 6) and has a molecular mass of approximately 75 kDa. The protein structure includes several protein-binding domains and is highly homologous to the mouse Cd2ap (figure 2). At the NH_2 terminus, three SRC homology 3 (SH3) domains are localized followed by a proline-rich region containing SH3-binding domains. Kirsch *et al* found four putative actin-binding sites at the COOH terminus (aa 534-538/599-603/610-614 and 631-635) similar to the LKKTET motifs found in a number of actin-binding partners [4]. Direct interaction between F-actin and Cd2ap was proven by actin filament precipitation assays. The COOH-terminal domain (aa 331-637) of Cd2ap was found to bind to F-actin directly [15]. Finally, CD2AP also contains a super-coiled domain (SC, aa 597-639) through which the protein forms homodimers [4].

As yet, only one heterozygous CD2AP mutation detected in two patients with primary FSGS has been reported. In these patients, the nucleotide changes (GC>CT) affected the splice acceptor of exon 7 leading to a reduced expression level of CD2AP in lymphocytes [3]. In this report, a novel homozygous mutation is presented. Moreover, we were able to show that the CD2AP interaction with filamentous (F-actin) was affected by this homozygous mutation and that the mutated allele is not expressed in lymphocytes.

Table 1 CD2AP/Cd2ap interacting proteins

Protein	Function	Reference
Anillin	Actin-binding protein involved in cytokinesis	Monzo *et al.* [6]
ASAP1	Membrane trafficking and cytoskeletal remodeling	Liu *et al.* [7]
CAPZ / CP	Regulates the growth of the actin filament	Hutchings *et al.* [8] and Bruck *et al.* [9]
c-Cbl	Negative regulator of the tyrosine kinase signaling pathway	Kirsch *et al.* [10], Cormont *et al.* [11], and Kobayashi *et al.* [12]
CD2	T-cell polarization	Dustin *et al.* [1]
CFBP	Internalization and down-regulation of the EGF receptor	Konishi *et al.* [13]
Cortactin	Regulation of receptor-mediated endocytosis	Lynch *et al.* [14]
Endophilin	Growth factor receptor endocytosis	Lynch *et al.* [14]
F-actin	Major component of the cell cytoskeleton	Lehtonen *et al.* [15]
Flt-1 (VEGFR-1)	VEGF receptor 1, involved in angiogenesis	Kobayashi *et al.* [12]
Grb^2	Regulates complex formation of proteins involved in growth signaling pathways	Kirsch *et al.* [4]
NPHS1	Key component of the podocyte slit diaphragm	Palmen *et al.* [16] and Shih *et al.* [17]
NPHS2	*Establishment of the podocyte slit diaphragm*	Schwartz *et al.* [18]
$p130^{Cas}$	Linking the actin cytoskeleton to the extracellular matrix	Kirsch *et al.* [4]
PI3K	Lipid kinase that controls complex cellular programs	Kirsch *et al.* [4] and Huber *et al.* [19]
PKD2	Involved in preserving the kidney tubular epithelial cells	Lehtonen *et al.* [5]
Rab4	Involved in early endocytosis	Cormont *et al.* [11]
Synaptopodin	Cell shape and motility	Huber *et al.* [20]
Fyn, Scr, Yes	Tyrosine kinases	Kisch *et al.* [4]

Interacting domains of CD2AP/Cd2ap with the proteins enlisted above are illustrated in figure 1, with the exception of NPHS2, of which the specific domain is not known. The interaction between these proteins and CD2AP/Cd2ap are proven by *in vitro* interaction studies.

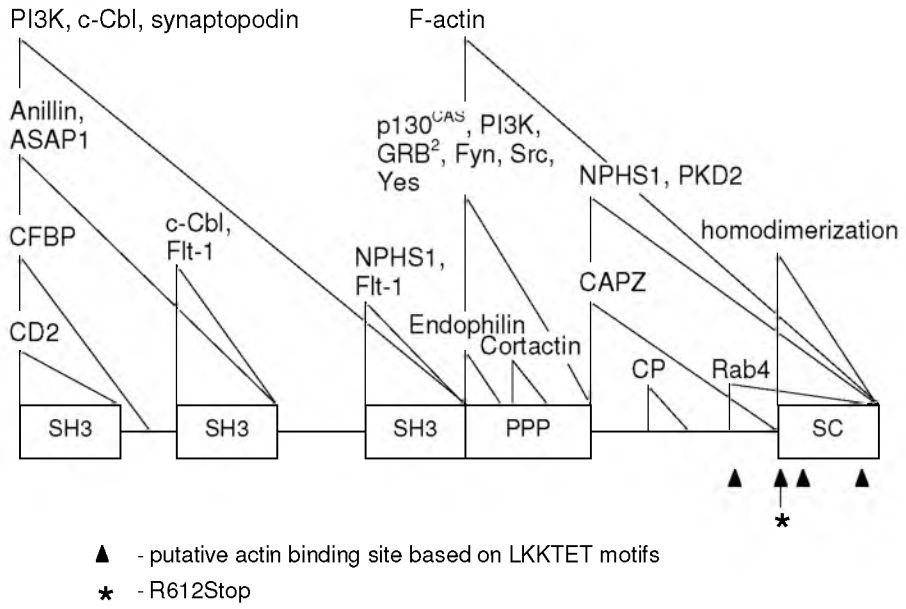

Figure 1 Schematic representation of the CD2AP/Cd2ap protein and its interacting partners. CD2AP/Cd2ap contains three SRC homology 3 (SH3) domains followed by a proline-rich region (P). At the COOH terminus, a super coiled domain (SC) is found. CD2AP/Cd2ap uses this domain for homodimerization [4]. The putative actin binding sites [4] and the location of the mutation described in this chapter are shown. The defined proteins are proven to interact *in vitro* with CD2AP and/or Cd2ap.

Methods

Study population

The molecular genetic study was performed in a group of 20 non-familial histologically proven FSGS patients. The study was performed with informed consent of the patients and their family members. The clinical data of the patient with the homozygous *CD2AP* mutation are listed under results.

Molecular genetic study

Genomic DNA was isolated from peripheral blood leukocytes using a salting-out method. Amplification of the *CD2AP* gene (GenBank accession numbers NM_001767 and NT_011109) was performed by PCR using primers in the intron regions flanking the exons. The PCR products were analyzed by DNA sequencing (Dye Terminator Cycle Sequencing, PE Applied Biosystems, Foster City, CA, USA). The genomic DNA from 50 healthy controls was used to confirm mutations and to exclude DNA-polymorphisms.

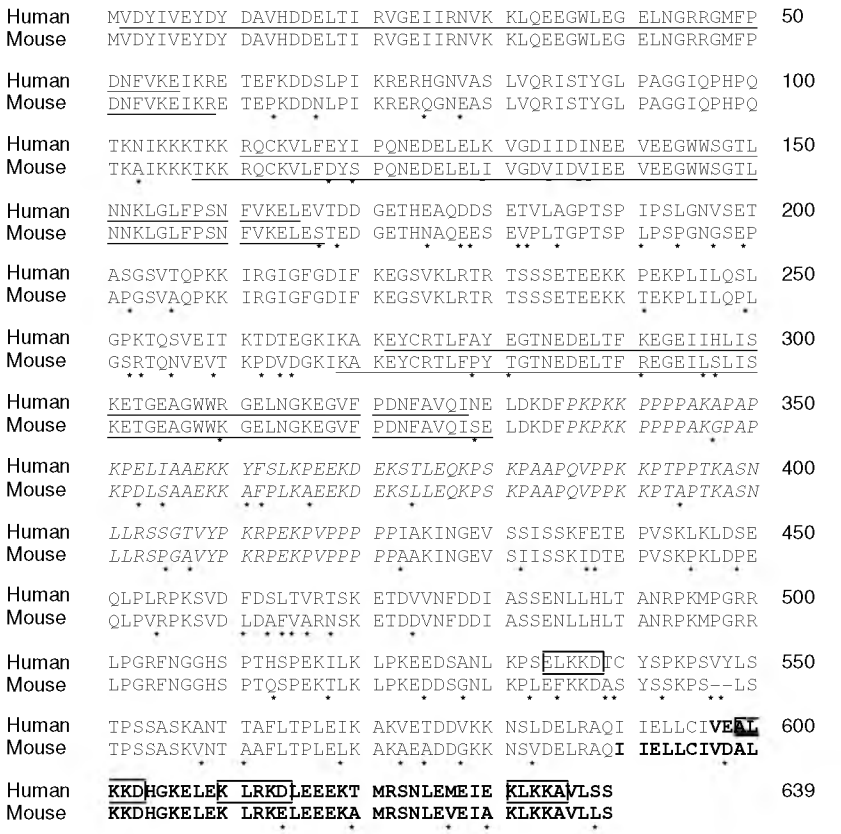

Figure 2 Alignment of human CD2AP [4] and mouse Cd2ap [5] showing high homology. The asterisks indicate non-matching amino acids. The different domains are shown (SH3 domain underlined, praline-rich region in italic, and the SC domain in bold), and the putative actin binding sites are in rectangles.

Actin-binding assay

The actin-binding assay was performed as described before by Kaplan *et al* [23]. In short, we developed expression constructs holding the entire coding region of CD2AP for *in vitro* transcription. The wildtype cDNA of CD2AP was cloned into pBluescript II SK (+) (Stratagene, La Jolla, CA, USA) by PCR and the mutant was created using the QuickChange II Site-Directed Mutagenesis kit (Stratagene).

In vitro translation of the wild-type and mutant CD2AP was accomplished according to the user's manual of the TnT-Coupled Reticulocyte Lysate kit (Promega, Madison, WI, USA). To study the interaction of the non-labeled *in vitro*-translated products with F-actin, we first polymerized G-actin under the following conditions: incubating G-actin (1.3 µM) in a 40 µl reaction buffer containing (in mM): KCl (100), $MgCl_2$ (2), ATP (0.5), DTT (dithiothreitol) (0.5), and Tris (10, pH 7.4) for 1 h at room temperature. Later, we added 5 µl (out of 25) of the *in vitro*-translated products and incubated for an additional hour. The samples were subsequently centrifuged at 100 000 g for 60 min at 21 °C. The supernatant was removed and used for gel electrophoresis. The pellet was resuspended in the initial volume of 40 µl and also used for gel electrophoresis on a 10% acrylamide gel. The samples were

transferred to Immobilon Transfer Membranes (Millipore, Billerica, MA, USA) and probed by a rabbit polyclonal anti-CD2AP antibody (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and by a mouse monoclonal anti-actin antibody (diluted 1:10 000; MP Biomedicals, Illkirch, France). Secondary antibodies were AP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (diluted 1:10 000, Tropix, Applied Biosystems, Foster City, CA, USA) and protein bands were visualized using CDP-*Star* chemiluminescent substrate (Tropix, Applied Biosystems). Finally, bands were quantified by an image analysis system (Leika, Cambridge, UK) to determine the percentage of binding protein.

CD2AP expression in lymphocytes

Lymphocytes were isolated from whole blood 4.5x diluted with phosphate-buffered saline using Ficoll Paque (1.078 g/ml; Pharmacia, Piscataway, NJ, USA) and centrifugation at 540g for 30 min. The interface was removed and washed 4 times with phosphate-buffered saline for 15 min at 300g. The cells were lysed on ice for 30 min in RIPA buffer containing protease inhibitors PMSG (pregnant mare serum gonadotrophin) (100 µg/ml), aprotinin (5 µg/ml) and sodium orthovanadate (1 mM). The cells were subsequently sonificated three times for 10 s and centrifuged at 13 000 g and 4°C for 20 min.

Twelve microgram of total protein was separated on a 12% acrylamide gel and transferred to Protran Nitrocellulose Membranes (Schleicher & Schuell, Dassel, Germany). The membranes were probed by a rabbit polyclonal anti-CD2AP antibody (diluted 1:200; Santa Cruz Biotechnology) followed by a horseradish peroxidase-conjugated secondary antibody goat anti-rabbit IgG (diluted 1:2 000; DakoCytomation, Glostrup, Denmark). As a control for protein loading a mouse monoclonal anti-GAPDH (diluted 1:10 000; Abcam, Cambridge, UK) was used followed by a horseradish peroxidase-conjugated secondary antibody goat anti-mouse IgG (diluted 1:1 000; DakoCytomation). Protein bands were visualized using the ECL+ detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Results

Case report

A male child of consanguineous parents of Mediterranean ancestry with no family history of kidney disease was born prematurely at gestational age 35.3 weeks by caesarean section because of fetal distress. His birth weight was 2746 g and Apgar score was 9/10.

At the age of 10 months, the patient was evaluated because of a failure to thrive (height 68 cm (-2.5 s.d.), weight 6120 g (weight to height < -2.5 s.d.). His clinical examination revealed a pale skin color, enlarged liver (2 cm below the right costal margin), and no peripheral edema. Blood pressure was elevated (124/57 mm Hg). Laboratory examination demonstrated microcytic anemia (hemoglobin 5.8 mmol/l and mean corpuscular volume 78 fl), normal serum creatinine (30 µmol/l), and low serum albumin 18 g/l. Anemia was successfully treated with iron and erythropoietin. Microscopic urine analysis showed microscopic hematuria (red blood cells > 30/hpf with > 60% dysmorphic red blood cells) and

the presence of hyaline cylinders. Urinary protein excretion was severely elevated (total protein 10.5 g/l, protein/creatinine 60 g:10 mmol). Laboratory values during follow-ups are summarized in figure 3a. Renal biopsy was performed and demonstrated global glomerular sclerosis in 4/10 glomeruli; 1/10 glomerulus showed a palisade conformation of the visceral epithelium, suggestive of collapsing type glomerulosclerosis; the other 5/10 glomeruli showed mesangial proliferation and matrix expansion and hypertrophic visceral epithelium. Electron microscopic examination of two glomeruli showed mesangial matrix expansion and mild effacement of the podocyte foot processes (figure 3b and 3c). Immunosuppressive therapy was deemed to be unuseful, considering the lesions observed in the renal biopsy.

Antiproteinuric therapy with enalapril and diuretics resulted in an initial reduction of proteinuria by >50%. Over the next year, a progressive deterioration of the glomerular filteration rate was noted. At 2 years and 10 months, the glomerular filtration rate estimated by Schwartz formula was 24 ml/min/1.73 m2, blood pressure was well controlled and the clinical condition of the patient was satisfactory. At the age of 3 years, the patient suddenly presented with severe hypertension, acute respiratory insufficiency, cardiac decompensation, and acute renal failure. This episode was preceded by several days of diarrhea and fever. Temporary ventilatory support was instigated, and peritoneal dialysis was commenced. Blood culture revealed *Salmonella Enteritides*. Renal failure did not recover. At the age of 5 years, the patient has undergone a successful post-mortal renal transplantation. No recurrence of proteinuria or anemia was observed. Blood analysis showed no lymphopenia. Before renal transplantation, the patient was treated with recombinant growth hormone to improve his growth velocity; however, after renal transplantation, his growth is still insufficient despite normal renal function and low steroid dose.
Urine examination of both parents revealed no proteinuria.

Molecular genetic study
We analyzed a group of 20 histologically proven FSGS patients for the presence of CD2AP mutations. In one patient, we found a mutation in exon 18 (figure 4). It concerns a homozygous substitution at position 1834 C>T (R612Stop), resulting in a premature stop codon situated at the COOH terminus. This premature stop codon leads to a truncation of approximately 4% compared to the native CD2AP protein. Both parents were proven to be heterozygous for this mutation. The mutation was not found in our control group of 50 healthy individuals.

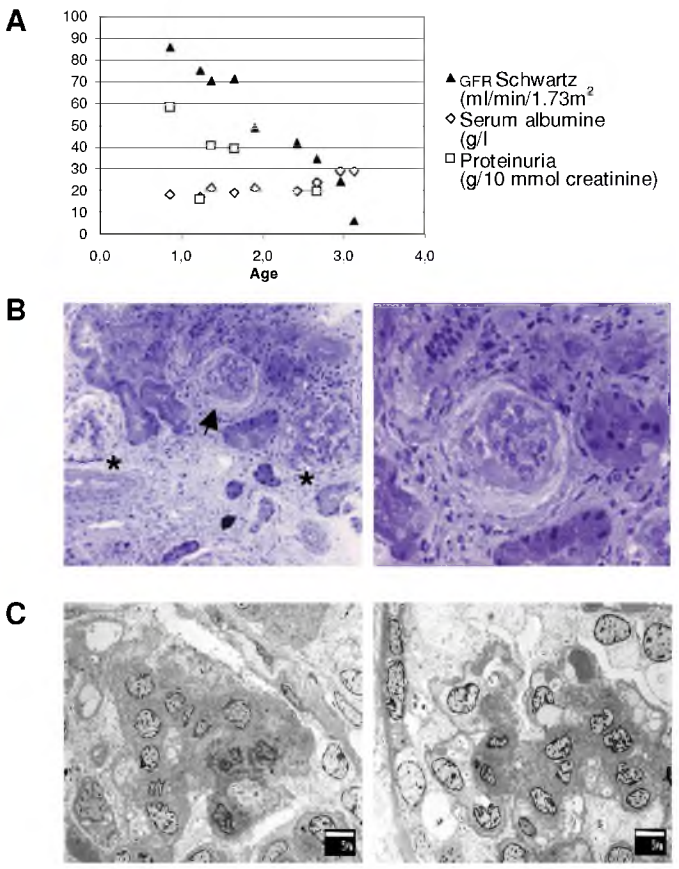

Figure 3 Clinical data and renal biopsy of the patient with the homozygous CD2AP mutation. **A.** Clinical data during follow-up showing a decline of glomerular filtration rate Schwartz and proteinuria and a slight elevation of serum albumin. **B.** Light microscopy (toluidine blue staining of semi-thin-sectioned plastic embedded biopsy). In the centre of the left panel, one glomerulus shows a segmental lesion (arrow), while the two other glomeruli show several degrees of mesangial proliferation (asterisks). The right panel displays a higher magnification of the glomerulus with segmental lesion (original magnification x20 (left) and x40 (right)). **C.** Electron microscopy. Left picture shows a segmental sclerosis lesion. On the right, an example of mesangial proliferation accompanied by mild increase of mesangial matrix is shown.

Actin Binding Assay

To investigate whether the detected CD2AP mutation affects the interaction with actin, we performed an actin-binding assay. *In vitro*-translated CD2AP wild-type and mutant proteins were incubated with polymerized F-actin. After incubation, F-actin and the possible bound proteins were pelleted by centrifugation.

Western blot analysis shows a successful polymerization of globular-actin (G-actin) to F-actin, since a fewer amount of G-actin is present in the supernatant. Non-polymerized G-actin (incubated in water) stays in solution after centrifugation (figure 5a). In the absence of actin, the *in vitro* translated CD2AP protein also stays in the supernatant after centrifugation (figure 5b).

Densitometric analysis of the wild-type CD2AP revealed an almost equal distribution between the supernatant (not bound) and the pelleted (bound) fraction. More accurately, the amount of wild-type CD2AP which was bound to F-actin and formed a pellet, was approximately 40% (figure 5c).

The mutant R612Stop protein shows, as predicted, a lower protein band (estimated molecular mass of approximately 70 kDa) compared to the wild-type (75 kDa). The amount of the R612Stop mutant protein bound to actin was reduced to approximately 6%. Consequently, a higher amount of the mutant protein is seen in the supernatant (figure 5c). This result provides evidence that the 4% truncation of the CD2AP protein at the COOH terminus owing to the mutation results in a disturbed interaction with F-actin. The actin-binding assay was performed in duplicate and showed identical results.

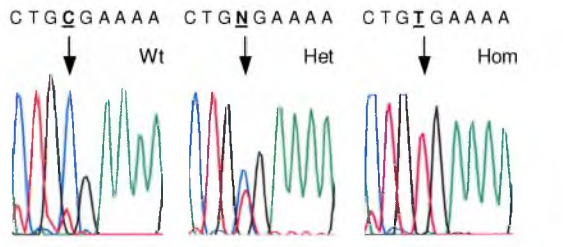

Figure 4 Sequence chromatogram of the CD2AP mutant. The arrows show the 1834 C>T substitution of the patient (Hom) and in the parents (Het) in comparison with the wild-type (Wt).

CD2AP expression lymphocytes

The patients with FSGS described by Kim *et al* showed a heterozygous CD2AP mutation with a reduced expression level of CD2AP in immortalized B-lymphocytes [3]. To determine the *in vivo* expression of CD2AP in the patient and his heterozygously mutated parents, we performed Western blot analysis on lysates from lymphocytes and compared the level of CD2AP expression with lymphocyte lysates from a control individual. Membranes were incubated with a polyclonal rabbit anti-CD2AP antibody and the results are seen in figure 5d.

The immunoblot shows no CD2AP expression in the patient carrying the R612Stop mutation homozygously (Hom). Furthermore, there is no difference in CD2AP expression between the parent and the control individual given the equal GAPDH presence. Both the control (Wt) and the parent (Het) show a band of the same molecular mass, while the truncated CD2AP protein has a predicted molecular mass of approximately 70 kDa. These results indicate that the mutated allele is not expressed.

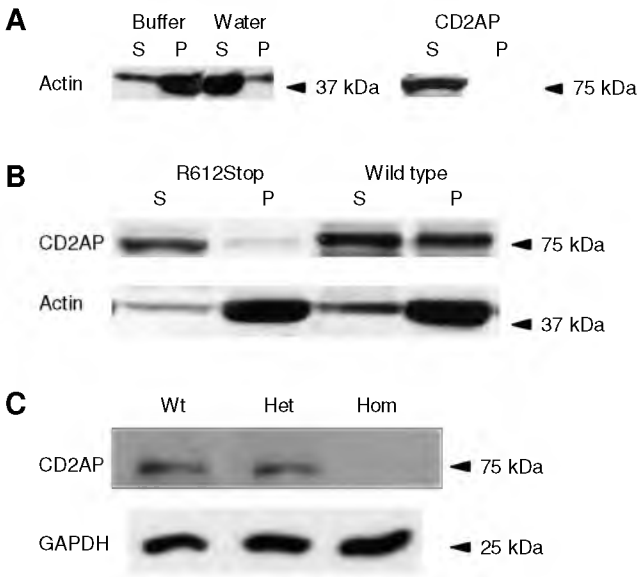

Figure 5 COOH-terminal truncation of 4% results in a disturbed interaction of CD2AP with F-actin and no CD2AP expression in lymphocytes. **A.** G-actin polymerizes successfully to F-actin under physiological conditions (lanes marked buffer) and forms a pellet (P) after centrifugation. In water non-polymerized G-actin stays in the supernatant (S) fraction, and pellets at a low level (lanes marked water). **B.** *In vitro* translated CD2AP (5 of 25 µl, incubated in polymerization buffer without actin) is found in the supernatant fraction. **C.** *In vitro* translated wild-type CD2AP shows an almost equal distribution between the fractions, with 40% of the protein co-precipitated with F-actin (upper panel, lanes marked wild type). Protein R612Stop exhibits a slightly lower molecular mass because of the premature stop codon, and is found in the supernatant with only a negligible amount in the pellet (6%). The lower panel shows a successful polymerization of F-actin. **D.** CD2AP levels in a control sample (Wt), the patient (Hom), and one of the parents carrying the mutation R612Stop heterozygously (Het). Cell lysates from lymphocytes were immunoblotted with anti-CD2AP (upper panel) and with anti-GAPDH as a control for protein loading.

Discussion

In this report we describe a novel homozygous mutation in the adaptor-type protein CD2AP in a patient with FSGS. The mutation (R612Stop) results in a premature stop codon and a truncation of 4%, such that aa 613-639 of the protein are lacking. An actin binding assay, in which the *in vitro* translated mutated proteins were incubated with filamentous actin, showed a dramatic reduction of actin binding due to the R612stop mutation.

The functional consequences of CD2AP dysfunction/absence have been investigated by studying a mouse model [2]. The $Cd2ap^{-/-}$ mice (the mouse homolog of CD2AP) die at an age of 6-7 weeks because to severe kidney disease involving the glomerulus. Already in 1-week-old knockout mice, glomeruli show an increase in size and cellularity, and electron microscopic examination showed foot process effacement. At 2 weeks of age, almost all glomeruli were affected and mesangial deposits were detected. By 4 weeks, glomeruli were

sclerotic, capillary loops were extended, and an increase of mesangial deposits was seen [2]. $Cd2ap^{+/-}$ mice did not exhibit proteinuria, but showed glomerular lesions at 9 month of age, similar to the ones in 3 to 4-weeks-old $Cd2ap^{-/-}$ mice. Some lesions were similar to FSGS [3]. Because of the phenotype of $Cd2ap^{+/-}$ mice, primary FSGS patients have been tested for CD2AP mutations. One mutation reported in the literature affects the splice acceptor of exon 7 on one allele that replaces two nucleotides, GC with CT. This change results in aberrant splicing between exon 4 and 18, and the predicted protein product would lack more than 80% of the CD2AP protein. This mutation yields to reduced CD2AP expression, and no expression of the truncated allele, as investigated by immunoblotting CD2AP isolated from lymphocytes. In these patients CD2AP haploinsufficiency resulted in FSGS [3].

In this study we present a homozygous mutation in CD2AP (R612Stop), not described before. The predicted mutated protein would lack only 4% of the COOH-terminal end of CD2AP. This effect of the mutation in heterozygous state is subtle, since the heterozygous parents are not clinically affected. In the heterozygous state, the CD2AP expression level is similar compared to the wild-type expression level as shown by immunoblotting (Figure 5d). The presence of the wildtype sized and not the truncated sized CD2AP protein in the heterozygous parents strongly suggests that the mutated allele hardly influences expression from the wild-type allele. In the homozygous state, the effect of the R612Stop mutation is more dramatic. Analysis of the *in vivo* expression of CD2AP in the lymphocytes of the patient showed a complete absence of CD2AP expression (Figure 5d). Furthermore, we clearly showed that the COOH-terminal truncation of the CD2AP protein has serious consequences on binding F-actin as showed by the *in vitro* actin-binding system: only 6% of the truncated CD2AP protein was bound to F-actin compared to 40% of the wild-type protein. Direct interaction between F-actin and the COOH terminus of Cd2ap (aa 331-637) has been proven by precipitation assays before [15], and in CD2AP, four putative actin binding sites were found similar to the LKKTET motifs found in a number of actin binding partners [4]. The homozygous R612Stop mutation is located in the second putative actin-binding site in the super-coiled domain. The disturbance in actin binding, as showed by the actin-binding assay, provides evidence that the 27 amino acids COOH-terminal tail that is lacking due to the stop mutation is required for actin binding.

The R612Stop mutation may also affect (1) homodimerization that takes places via the super-coiled domain of CD2AP or (2) binding to other proteins like NPHS1, Rab4 or PKD2 (figure 1 and table 1). These proteins all bind CD2AP at a specific domain in which the homozygous mutation is situated. The R612Stop mutation has a subtle effect in heterozygous state, in contrast to the exon 7 splice acceptor mutation GC>CT (Kim *et al*) that has severe consequences when found heterozygous [3]. This difference is most probably caused by the level of truncation of the CD2AP protein. The 80% truncation owing to the splice acceptor mutation will have a more dramatic effect on CD2AP expression and function than the 4% truncation resulting from the R612Stop mutation. Apparently, for this reason, the effect of the R612Stop mutation is only observed when both alleles are affected.

In this paper, we have described a novel *CD2AP* mutation (R612Stop Hom) in a patient with primary FSGS. Although the first described mutation in *CD2AP* by Kim *et al*

affected only one allele in two FSGS patients, we would like to stress that not all heterozygous *CD2AP* mutations cause kidney diseases as shown in our family. Development of FSGS clearly depends on the severity of the mutation.

References

1. Dustin ML, Olszowy MW, Holdorf AD *et al.* A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* 1998; **94:** 667-677.

2. Shih NY, Li J, Karpitskii V *et al.* Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* 1999; **286:** 312-315.

3. Kim JM, Wu H, Green G *et al.* CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. *Science* 2003; **300:** 1298-1300.

4. Kirsch KH, Georgescu MM, Ishimaru S *et al.* CMS: an adapter molecule involved in cytoskeletal rearrangements. *Proc Natl Acad Sci U S A* 1999; **96:** 6211-6216.

5. Lehtonen S, Ora A, Olkkonen VM *et al.* In vivo interaction of the adapter protein CD2-associated protein with the type 2 polycystic kidney disease protein, polycystin-2. *J Biol Chem* 2000; **275:** 32888-32893.

6. Monzo P, Gauthier NC, Keslair F *et al.* Clues to CD2-associated protein involvement in cytokinesis. *Mol Biol Cell* 2005; **16:** 2891-2902.

7. Liu Y, Yerushalmi GM, Grigera PR *et al.* Mislocalization or reduced expression of Arf GTPase-activating protein ASAP1 inhibits cell spreading and migration by influencing Arf1 GTPase cycling. *J Biol Chem* 2005; **280:** 8884-8892.

8. Hutchings NJ, Clarkson N, Chalkley R *et al.* Linking the T cell surface protein CD2 to the actin-capping protein CAPZ via CMS and CIN85. *J Biol Chem* 2003; **278:** 22396-22403.

9. Bruck S, Huber TB, Ingham RJ *et al.* Identification of a novel inhibitory actin-capping protein binding motif in CD2-associated protein. *J Biol Chem* 2006; **281:** 19196-19203.

10. Kirsch KH, Georgescu MM, Shishido T *et al.* The adapter type protein CMS/CD2AP binds to the proto-oncogenic protein c-Cbl through a tyrosine phosphorylation-regulated Src homology 3 domain interaction. *J Biol Chem* 2001; **276:** 4957-4963.

11. Cormont M, Meton I, Mari M *et al.* CD2AP/CMS regulates endosome morphology and traffic to the degradative pathway through its interaction with Rab4 and c-Cbl. *Traffic* 2003; **4:** 97-112.

12. Kobayashi S, Sawano A, Nojima Y *et al.* The c-Cbl/CD2AP complex regulates VEGF-induced endocytosis and degradation of Flt-1 (VEGFR-1). *FASEB J* 2004; **18:** 929-931.

13. Konishi H, Tashiro K, Murata Y *et al.* CFBP is a novel tyrosine-phosphorylated protein that might function as a regulator of CIN85/CD2AP. *J Biol Chem* 2006; **281:** 28919-28931.

14. Lynch DK, Winata SC, Lyons RJ *et al.* A Cortactin-CD2-associated protein (CD2AP) complex provides a novel link between epidermal growth factor receptor endocytosis and the actin cytoskeleton. *J Biol Chem* 2003; **278:** 21805-21813.

15. Lehtonen S, Zhao F, Lehtonen E. CD2-associated protein directly interacts with the actin cytoskeleton. *Am J Physiol Renal Physiol* 2002; **283:** 734-743.

16. Palmen T, Lehtonen S, Ora A *et al.* Interaction of endogenous nephrin and CD2-associated protein in mouse epithelial M-1 cell line. *J Am Soc Nephrol* 2002; **13:** 1766-1772.

17. Shih NY, Li J, Cotran R *et al.* CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am J Pathol* 2001; **159:** 2303-2908.

18. Schwarz K, Simons M, Reiser J *et al.* Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J Clin Invest* 2001; **108:** 1621-1629.

19. Huber TB, Hartleben B, Kim J *et al.* Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol Cell Biol* 2003; **23:** 4917-4928.

20. Huber TB, Kwoh C, Wu H *et al.* Bigenic mouse models of focal segmental glomerulosclerosis involving pairwise interaction of CD2AP, Fyn, and synaptopodin. *J Clin Invest* 2006; **116:** 1337-1345.

21. Schiffer M, Mundel P, Shaw AS *et al.* A novel role for the adaptor molecule CD2-associated protein in transforming growth factor-beta-induced apoptosis. *J Biol Chem* 2004; **279:** 37004-37012.

22. Peters I, Tossidou I, Achenbach J *et al.* IGF-binding protein-3 modulates TGF-beta/BMP-signaling in glomerular podocytes. *J Am Soc Nephrol* 2006; **17:** 1644-1656.

23. Kaplan JM, Kim SH, North KN *et al.* Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* 2000; **24:** 251-256.

Chapter 6

Bigenic heterozygosity and the development of steroid-resistant focal segmental glomerulosclerosis

Focal segmental glomerulosclerosis (FSGS) is a major cause of steroid resistant nephrotic syndrome in childhood with a central role for the podocytes in the pathogenesis. Mutated proteins expressed in podocytes cause proteinuria. The role of combined gene defects in the development of FSGS is less clear. We analysed seven podocyte genes known to cause proteinuria and FSGS in a group of twenty non-familial childhood-onset steroid-resistant FSGS patients. These genes include *NPHS1, NPHS2, ACTN4, CD2AP, WT-1,TRPC6,* and *PLCE1*. We also screened for the mitochondrial A3243G DNA transition associated with the MELAS syndrome (mitochondrial Myopathy, Encephalopathy, Lactic acidosis, and Stroke-like episodes), and occasionally FSGS. No mutations were found in the *ACTN4* and *TRPC6* genes, and no mitochondrial A3243G DNA transition was found in our group of patients. Two patients showed mutations in the *CD2AP* gene, one combined with a *NPHS2* mutation. A tri-allelic hit was found in a patient carrying compound heterozygous *NPHS2* mutations and a heterozygous *NPHS1* mutation. In another patient a *de novo WT-1* mutation was found combined with a heterozygous *NPHS1* mutation, and finally, one patient showed a single heterozygous *PLCE1* mutation. In our rather small group of 20 steroid-resistant FSGS patients, four mutations in podocyte genes were found that could explain the pathology. Our data suggests that combined gene defects in podocyte genes may play a role in the development of FSGS.

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Introduction

Focal segmental glomerulosclerosis (FSGS) is a major cause of steroid resistant nephrotic syndrome in childhood, accounting for about 75% of patients and frequently leading to end stage renal disease. The aetiology of primary FSGS remains unknown in most cases. The recurrence of the disease after renal transplantation in approximately one third of the patients, suggests the presence of a "circulating FSGS factor", which until now has not been identified [1].

Molecular studies in humans and mouse models revealed a central role of glomerular podocyte damage in the development of FSGS [2]. A growing number of proteins, expressed by podocytes, contributing to the structure of the slit-diaphragm (nephrin, podocin, CD2AP) and/or function of the cytoskeleton (alpha-actinin 4) of the podocyte, cause proteinuria when mutated [3-6]. Mutations in the WT1 transcription factor [7] and in TRPC6 [8], a calcium-permeable cation channel, also lead to abnormal podocyte function and proteinuria. More recently, mutations in *PLCE1*, encoding a phospolipase involved in the initiation of a cascade of cellular processes resulting in cell growth, cell differentiation, and gene expression, were found in familial FSGS patients [9]. The mitochondrial A3243G transition is mainly associated with the MELAS syndrome (mitochondrial Myopathy, Encephalopathy, Lactic acidosis, and Stroke-like episodes) [10], but is also found in FSGS patients possibly with or without maternally inherited diabetes and/or sensorineural hearing loss [11,12].

It might be suggested that the degree of podocyte dysfunction determines whether the patient would develop congenital nephrotic syndrome or nephrotic syndrome due to FSGS later in life. Studies in mice demonstrated that bigenic heterozygosity in podocyte genes (*CD2AP*$^{+/-}$ with *Fyn*$^{+/-}$ or *Synpo*$^{+/-}$ mice) led to the development of proteinuria and FSGS-like renal damage, while isolated haploinsufficiency did not cause renal disease [13]. Whether combined defects in podocyte genes play a role in the development of human FSGS is less clear. In this study, we have therefore investigated the occurrence of mutations of seven podocyte genes in a group of non-familial childhood-onset steroid-resistant FSGS-patients.

Subjects and methods

Patients

Twenty patients, aged 10-162 (average 73) months, 14 males, with steroid resistant nephrotic syndrome due to biopsy proven FSGS were analysed. Steroid resistance was defined as persistent proteinuria after 6 weeks of prednisone treatment (60 mg/m^2/day). The relevant clinical data of the patients are provided in table 1.

Molecular Genetic studies

DNA was isolated from peripheral blood leukocytes and urine sediments were collected from non-transplanted patients to isolate DNA using a commercially available DNA isolation kit (Puregene™ DNA isolation kit, Gentra systems, MN).

Amplification of the *NPHS1* (GenBank accession number AF190637-AF035835), *NPHS2* (AJ279246-AJ279253), *CD2AP* (AF164377/NT_007592), *WT-1* (AH003034), *ACTN4* (NM_004924.2), *TRPC6* (NP_004612), and *PLCE1* (NM_016341/NT_030059) exons was performed by PCR. Primer data are available on request. The 5'-UTR region of *NPHS1* was also analyzed. The PCR products were analyzed by DNA sequencing (Dye Terminator Cycle Sequencing, PE Applied Biosystems, Foster City, CA, USA). The genomic DNA from 150 healthy ethnically matched control individuals was used to confirm novel mutations.

Screening for the mtDNA A3243G transition (MELAS mutation) was performed by PCR using the following primers: 5'-CAACTTAGTATTATACCCACAC-3' and 5'-ATTAGAATGGGTACAATGAGGA-3', leading to a PCR product of 162 bp. PCR products were subsequently digested overnight with *ApaI* restriction enzyme. The presence of the A3243G mutation results in an additional restriction site on position 104 bp of the PCR product. The restriction fragments were analyzed on a 1% agarose gel containing ethidium bromide to visualize the DNA. Due to the heteroplasmic state of the mitochondrial mutation and the differences in threshold values of tissues, mutation analysis was performed using DNA from peripheral blood leukocytes and from urine sediment.

Results

All patients presented with steroid resistant FSGS. Treatment regiment after renal biopsy and response to this treatment are listed in table 1.

Not one patient had a mutation in the *ACTN4* or *TRPC6* gene and no MELAS mutation in the mtDNA was found. Four patients (20%) had mutations in *WT1*, *NPHS1*, *NPHS2* and/or *CD2AP* genes that could explain the pathology (see table 2 and figures 1 and 2). In addition, two patients showed three heterozygous *PLCE1* mutations (see table 2). Short clinical summaries and the results of DNA analysis of the four patients in which the disease-causing mutations have been established are provided below.

Table 1 Clinical data of the patients with non-familial steroid-resistant FSGS

		Data at admission						
Pt	**Sex**	*Age at onset* (months)	*Proteinuria* (g/l)	*Serum albumin* (g/l)	*Serum creatinine* (µmol/l)	*Immuno suppressive treatment*	**Response to treatment**	**FSGS classification**
1	M	42	6.0	26	79	csA, Pred	no	NOS, adv. Col.
2	F	36	5	16	21	Cph	no	mild Col.
3	F	103	14.5	23	51	csA, Pred	no	early NOS
4	M	10	10.5	18	30	no	-	Mes. prol.
5	M	38	14	14	45	Cph, Pred	no	Col.
6	M	138	15.9	15	54	csA, Pred, MMF	PR	
7	F	22	49.4	19	141	csA, Pred	PR	
8	M	160	2.2	32	119	Pred	PR	
9	M	90	7.7	19	67	csA, Pred	PR	
10	F	49	6.3	30	36	csA, Pred	no	
11	M	12	19.2	20	35	csA, Pred	CR	
12	M	10	37.9	18	54	csA, Pred	CR	
13	F	94	4.6	28	112	csA, Pred	CR	Col.
14	M	64	3.2	25	43	csA, Pred	no	adv. NOS
15	F	128	11.1	18	51	csA, Pred	PR	
16	M	100	55	22	45	csA	no	na
17	M	72	5.8	11.5	269	no, †	-	adv. Per.
18	M	40	4.3	16	40	csA, Pred	PR	Cel.
19	M	162	2.9	38	130	Pred	CR*	early NOS
20	M	98	21.1	13.9	41	Pred, Renitec	CR	Mes. prol.

Pt = patient, M = male, F = female, csA = cyclosporin A, Pred = prednisone, MMF = mycophenolate mofetil, Cph = cyclophosphamide, CR = complete remission and PR = partial remission. FSGS Classifications: NOS = not otherwise specified, Col. = collapsing lesion, Tip = tip lesion, Per. = perihilar lesion, Cel. = cellular lesion, Mes. prol. = mesangial proliferation, adv. = advanced, na = not available

In this patient a short-standing decline of proteinuria under prednisone treatment was seen followed by an increase of proteinuria and deterioration of renal function.

Patient 1

A white male, the only child of two healthy parents, presented at the age of 3.5 years with an upper airway infection and oedema. Physical examination revealed severe peripheral oedema and ascites. Blood pressure was 120/83 mm Hg. Urinalysis showed proteinuria 6 g/l and microscopic haematuria. Serum creatinine was 79 µmol/l, albumin - 26 g/l and cholesterol 10.8 mmol/l. Complement factor C3 and C4 were normal. As six weeks of prednisone treatment 60 mg/m^2/day gave no improvement, renal biopsy was performed. Microscopic examination of renal tissue revealed FSGS. Patient 1 was treated with cyclosporin A, low dose of prednisone and enalapril without clinical response. End stage renal disease developed at the age of 8 years, when haemodialysis was initiated. Renal transplantation with a post-mortal graft was performed a half year later. Native kidneys remained *in situ*. Because of delayed graft function, renal biopsy was performed two weeks after transplantation and demonstrated profound acute tubular necrosis and podocyte foot effacement. Recurrence of FSGS in the renal graft was suggested and treated with plasma

exchange with only slight effect on renal function and proteinuria. At present he is 10 years old, has persisting nephrotic syndrome and pre-terminal renal failure.

Together with the earlier reported *NPSH2* (A208T) mutation of maternal origin [14], a heterozygous mutation of *CD2AP* (1488 G>A, M496I) (figure 1a), not described before, of paternal origin was found. The amino acid substitution in podocin replaces the aliphatic amino acid alanine by threonine, an amino acid with a sulphur containing side chain. Using a standard phosphorylation prediction site (Netphos 2.0), the presence of threonine in the mutated podocin protein may introduce an additional phosphorylation site for the protein kinase CKI and may therefore alter the secondary structure and possibly its oligomerization. The mutation is situated at the C-terminus responsible for interactions with nephrin and CD2AP [15] that may be affected by the mutation as well. The CD2AP (M496I) substitution results in the replacement of the sulphur containing amino acid methionine in the aliphatic amino acid isoleucine. The mutation is located between the proline rich region and the super-coiled domain at the C-terminus. Both parents had a normal serum creatinine and no proteinuria.

Table 2 Mutations detected in non-familial steroid-resistant FSGS patients

	WT-1			***NPHS1***			***NPHS2***			***CD2AP***			***PLCE1***		
P	*nt sub.*	*eff. mut*	*st. mut*	*nt sub.*	*eff. mut*	*st. mut*	*nt sub.*	*eff. mut*	*st. mut*	*nt sub.*	*eff. mut*	*st. mut*	*nt sub.*	*eff. mut*	*st. mut*
1							622 G>A	A208T	het	1488 G>A*	M496I	het			
2				791 C>G	P264R	het	413 G>A	R138Q	het						
							948 delT *	frame shift	het						
3	1228+5 G>A	splice	het	1126 C>G	L376V	het									
4										1834 C>T	R612X	hom			
5													1807 G>T *	E603X	het
6													3491 C>T *	T1164M	het
													3518 C>T *	S1177F	het

Patient (P) numbers 1-6 correspond with the numbers in table 1. * = Novel mutation, not reported in literature before, nt = nucleotide, sub. = substitution, eff. = effect, mut = mutation, st. = status, het = heterozygous, hom = homozygous

Patient 2

A white female presented at the age of 3 years with mild periorbital and pretibial oedema and slightly elevated blood pressure (120/70 mm Hg). Urinalysis revealed proteinuria 4-8 g/l and microscopic haematuria. Serum creatinine was in the normal range (21 µmol/l). During the 6 week treatment with prednisone (60 mg/m^2/day) the oedema became more pronounced. A biopsy was performed. Light microscopy showed collapse of some capillary loops with moderate sclerosis. Immunofluorescence demonstrated mild staining for IgM, C1q and C3 in the mesangium. Electronmicroscopy revealed fusion of the podocyte foot processes. Cyclophosphamide treatment gave no improvement. Her renal function gradually decreased. She received a post-mortal graft at the age of 5.5 years. The nephrotic syndrome did not yet recur.

Patient 2 presented a tri-allelic hit (figure 1b and c) [16]. The heterozygous *NPSH1* mutation (791 C>G, P264R) has been described before [16]. The amino acid substitution is situated in the extracellular domain of nephrin, in the third immunoglobulin motif. The cyclic amino acid proline is replaced by the basic amino acid arginine. Two heterozygous *NPSH2* mutations were also found in this patient. The first mutation (413G>A, R138Q), paternally inherited, is already known [4]. The amino acid substitution situated at the C-terminus involves the replacement of the basic amino acid arginine to the acidic amino acid glutamine. The second one (948 del T), has not been described before and results in a truncated protein and a premature stop codon 31 amino acids downstream. This mutation is situated in exon 8 and is responsible for an aberrant C-terminus of podocin. The mother had the *NPSH1* mutation and the *NPSH2* deletion. Two brothers of the patient were also screened for the presence of the *NPHS1*/*NPHS2* mutations (figure 2b). Both brothers were found to have the paternal R138Q *NPHS2* mutation and one also showed the maternal P264R *NPHS1* substitution. Both brothers, as well as both parents, had normal serum creatinine and no proteinuria.

Patient 3

Patient 3, a white female, presented at the age of 8.5 years with general malaise and abdominal pain. Physical examination was unremarkable; there was no peripheral oedema. Blood pressure was 120/80 mm Hg. Urinalysis revealed 14.5 g/l protein and 25-50 erythrocytes per high power field. Serum creatinine was 51 µmol/l, albumin - 23 g/l and cholesterol - 8.6 mmol/l. Complement factor C3 and C4 were normal. Hepatitis B, C and HIV serology was negative. Renal ultrasound was normal. She did not respond to the initial treatment with prednisone 60 mg/m^2/day during 6 weeks. Light microscopic examination of

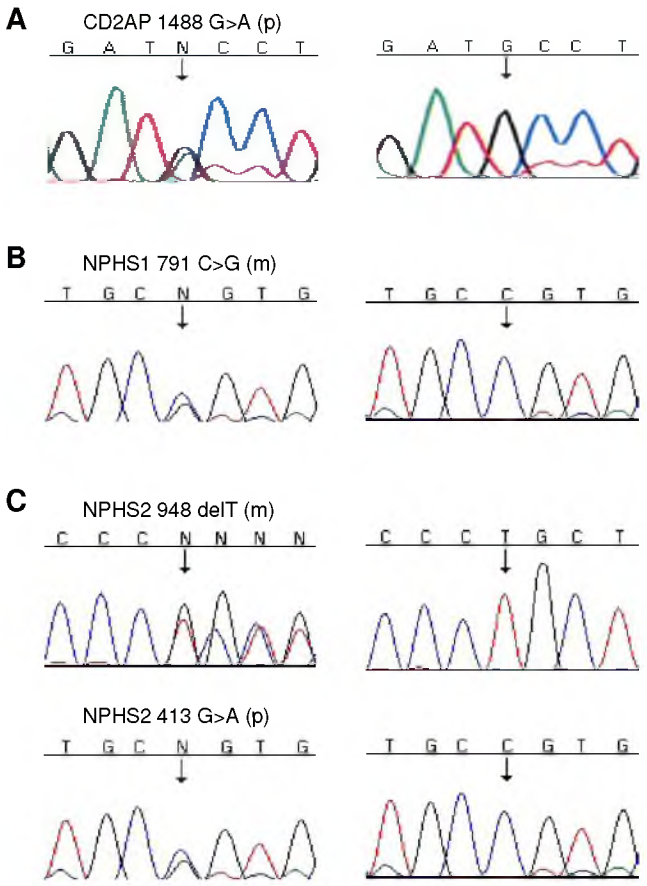

Figure 1 Sequence chromatograms of patients 1 and 2. The left panel shows the mutations in the patients, the right panel the wildtype sequence. **A.** The heterozygous 1488 G>A (M496I) substitution in the *CD2AP* gene found in patient 1 (paternal, (p)). This patient also carries a heterozygous *NPHS2* mutation (622 G>A, A208T, maternal) [14]. **B.** The arrow shows the maternally (m) inherited heterozygous 791 C>G (P264R) *NPHS1* substitution in patient 2. **C.** The maternally (upper) and paternally (lower) inherited mutations in *NPHS2*, 948 del T and 413 G>A (R138Q) respectively in patient 2.

renal tissue revealed FSGS in 4 glomeruli and slight mesangial hypercellularity in the other 10 glomeruli. Patient 3 was treated with cyclosporin A and low dose prednisone with an initially partial response: decrease of proteinuria to 1-2 g/l and increase of serum albumin to 34-37 g/l. The treatment with enalapril had to be discontinued because of the development of urticaria. Four years later she developed overt nephrotic syndrome despite the continuation of cyclosporin treatment and the addition of prednisone and angiotensin II receptor blocker. Renal function deteriorated rapidly with the development of end stage renal disease at the age of 14.5 years. After the initiation of haemodialysis, bilateral nephrectomy was performed because of the persistent nephrotic syndrome and severe hypertension. At the age of 16 years she received a post-mortal renal graft. Two-and-a-half years later she developed proteinuria (0.9 g/l) due to acute rejection without any signs of the recurrence of FSGS on

renal biopsy. Treatment by methyl-prednisolone was successful. The patient is currently 19 years old and has a well-functioning renal graft.

In addition to an earlier reported *de novo WT-1* mutation [14], she has a heterozygous mutation of *NPSH1* (1126C>G, L376V), already previously reported [3]. The heterozygous *NPHS1* mutation was of maternal origin. The mother had no symptoms.

Patient 4

This patient has a homozygous *CD2AP* mutation (1834 C>A, R612X) and is the subject of a separate report [17].

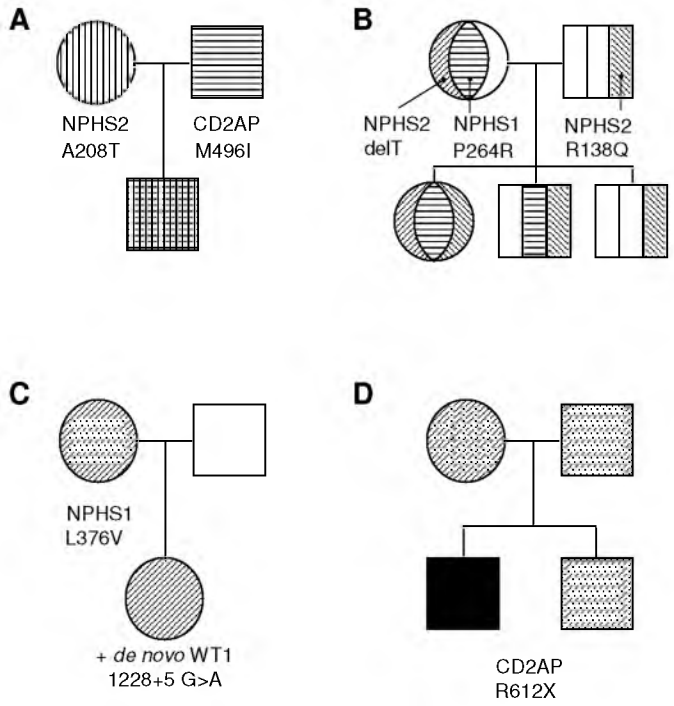

Figure 2 Family trees of the patients with mutations in one or more podocyte genes. **A.** Patient 1 with mutations in *NPHS2* (A208T) and *CD2AP* (M496I). **B**. The tri-allelic hit in patient 2 with mutations in the *NPHS1* (P264R) and *NPHS2* (delT/R138Q) gene. Parents and siblings did not show proteinuria. **C.** Inheritance of the *NPHS1* mutation (L376V) in patient 3 also carrying the *de novo WT-1* mutation 1228+5 G>A. **D.** The *CD2AP* mutation (R612Stop) in patient 4. (circle = female, box = male, dashed circles/boxes represent heterozygous mutations, the filled box a homozygous mutation)

Discussion

This is the first report on DNA analysis of seven podocyte genes in series of childhood-onset non-familial biopsy proven FSGS patients. Although this series of patients is rather small, it revealed interesting data suggesting that the combined haploinsufficiency in two podocyte genes might be responsible for the development of FSGS in humans.

Patient 1 had a combination of a previously reported maternal *NPHS2* mutation [14] and a novel paternal *CD2AP* mutation. Both parents carrying one of these mutations exhibited no renal disease. This finding is extremely interesting in light of the recent studies in mouse models of FSGS, demonstrating that heterozygous *CD2AP* mutations might sensitise podocytes to mutations of other genes (synaptopodin and Fyn proto-oncogene), while isolated heterozygous mutations in these genes did not result in the development of FSGS [13]. Our data suggests that in patients with FSGS and heterozygous *NPHS2* mutations, the additional analysis of *CD2AP* gene is warranted.

A tri-allelic digenic inheritance observed in our patient 2 has been previously reported in 5 patients in the series of Koziell *et al.* [16]. Both *NPHS2* mutations are situated in the nephrin binding domain (amino acid 125-385) [15]. Most probably the compound heterozygous state of the *NPHS2* mutations is causative for the development of FSGS. This is strengthened by the fact that one of the patients siblings carries the heterozygous R138Q *NPHS2* and P264R *NPHS1* mutations and did not show any proteinuria. Furthermore, the *NPHS1* mutation is not situated in the podocin interacting region of amino acid 1167-1256 [18].

In patient 3 the heterozygous *de novo* intron 9 splice-site mutation of *WT1* is probably responsible for the renal phenotype. This mutation is previously found in patients with the Frasier syndrome (nephrotic syndrome with FSGS, male pseudo-hermaphroditism, and a higher risk of developing gonadoblastoma). *WT1* intron 9 splice site mutations are responsible for a disturbed DNA binding capacity of this transcription factor [19]. The role of the additional maternal inherited *NHPS1* gene mutation is unclear. The *NPHS1* mutation (L376V) is first described in a patient with congenital nephrotic syndrome of the Finnish type (large placenta, proteinuria at birth with nephrotic syndrome appearing during the first weeks of life). The patient was homozygous for one mutation (P368S) and had the L376V mutation in only one allele [3].

In one patient (nr 5) a single *PLCE1* mutation was found (E603X). In the familial cases of nephrotic syndrome, two patients of one family out of six families investigated were found to have FSGS [9]. The biopsies of the other families revealed diffuse mesangial sclerosis (DMS). The histological differentiation between childhood-onset FSGS and infantile DMS is however not a sinecure. The single heterozygous mutation is most probably not causative for developing FSGS since both parents, and therefore also one of the carriers of this mutation, show no renal abnormalities. In a second patient (number 6) we observed two novel missense mutations in the *PLCE1* gene (T1164M and S1177F). Both mutations originated from the paternal allele and could therefore not explain the clinical pathology. Although *PLCE1* knockout mice did not show any nephrosis-like phenotype [9, 20], knocking

down the zebrafish ortholog of *PLCE1* showed foot process effacement and disorganization of the slit diaphragms. Nothing can be said about any possible effect on the glomerular filtration barrier when the zebrafish ortholog of *PLCE1* was knocked down only partially. It is not unlikely that, as in patient 1, future studies might reveal another gene to be involved in the pathogenesis of FSGS in patient 5 and maybe 6.

The meaning of a heterozygous mutation in developing a renal phenotype is not clear [21]. A single mutation in a recessive disorder is unable to induce a pathologic effect. The second mutation may have gone unnoticed or that another gene(s) may produce an additive effect. This additive effect could be the mutation in *CD2AP* in our first patient. The observed mutations in patient 1 is highly interesting in this respect. Podocin is a protein of 383 amino acids with a membrane domain and two cytoplasmic ends at the C- and N-terminus [4]. Podocin oligomers associate in a lipid raft at the podocyte membrane and clusters nephrin. CD2AP is an adaptor protein that functions as an integral member of the slit-diaphragm complex by interacting with podocin and nephrin and anchoring them to the actin cytoskeleton. Podocin interacts through its C-terminal end (amino acid 125 to 385) with CD2AP and nephrin [15]. The heterozygous *NPHS2* mutation in our patient was located inside the podocin interaction region and may impair the interaction with CD2AP. Two patients with FSGS and a mutation affecting the splice acceptor of exon 7 of *CD2AP* in one allele, described by Kim *et al* [6], resulted in a truncated protein. The paternally inherited *CD2AP* mutation in our patient is located between the proline rich region and the coiled-coil domain of the CD2AP protein. It is probable that the mutation in our patient affects the function of CD2AP to a lesser degree then in the patients described by Kim *et al* [6]. The *CD2AP* mutation alone is not responsible for the development of FSGS. The father with the heterozygous mutation of CD2AP had a normal serum creatinine and no proteinuria.

New genetic defects explaining FSGS will be revealed in the near future. For instance, missense mutations in *LAMB2* encoding laminin β2, may cause FSGS without eye abnormalities [22]. Analyses of *LAMB2* was not performed in our group since patients with this disorder have rarely no eye abnormalities. The knowledge of a genetic defect is important as aggressive treatment of the nephrotic syndrome can be avoided and the prognosis of the renal transplant can be predicted, although the finding of a genetic defect does not exclude the recurrence of nephrotic syndrome in the renal graft [23,24,25].

Our data demonstrate that combined genetic defects in podocyte genes may play a role in the development of FSGS in humans. Altered interactions between several podocyte proteins can make podocytes vulnerable for the “second hit” factors and result in genetic susceptibility of a subset of patients. Further studies of the numerous podocyte genes coming from human and animal studies will provide new insights in the pathophysiology of FSGS in humans.

References

1. Savin VJ, Sharma R, Sharma M *et al.* Circulating factor associated with increased glomerular permeability to albumin in recurrent focal segmental glomerulosclerosis. *N Engl J Med.* 1996; **334:** 878-883.

2. Woroniecki RP, Kopp JB. Genetics of focal segmental glomerulosclerosis. *Pediatr Nephrol.* 2007; **22:** 638-644.

3. Lenkkeri U, Mannikko M, McCready P *et al.* Structure of the gene for congenital nephrotic syndrome of the Finnish type (NPHS1) and characterization of mutations. *Am J Hum Genet* 1999; **64:** 51-61.

4. Boute N, Gribouval O, Roselli S *et al.* NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* 2000; **24:** 349-354.

5. Kaplan JM, Kim SH, North KN *et al.* Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* 2000; **24:** 251-256.

6. Kim JM, Wu H, Green G et al. CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. *Science* 2003; **300:** 1298-1300.

7. Ruf RG, Schultheiss M, Lichtenberger A *et al.* Prevalence of WT1 mutations in a large cohort of patients with steroid-resistant and steroid-sensitive nephrotic syndrome. *Kidney Int* 2004; 66: 564-570.

8. Winn MP, Conlon PJ, Lynn KL *et al.* A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science* 2005; **308:** 1801-1804.

9. Hinkes B, Wiggins RC, Gbadegesin R *et al.* Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. *Nat Genet.* 2006; **38:** 1397-1405.

10. Piechota J, Mroczek K, Bartnik E. MELAS as an example of a mitochondrial disease. *J Appl Genet.* 2001; **42:** 351-358.

11. Jansen JJ, Maassen JA, van der Woude FJ *et al.* Mutation in mitochondrial tRNA(Leu(UUR)) gene associated with progressive kidney disease. *J Am Soc Nephrol.* 1997; **8:** 1118-1124.

12. Kurogouchi F, Oguchi T, Mawatari E *et al.* A case of mitochondrial cytopathy with a typical point mutation for MELAS, presenting with severe focal-segmental glomerulosclerosis as main clinical manifestation. *Am J Nephrol.* 1998; **18:** 551-556.

13. Huber TB, Kwoh C, Wu H *et al.* Bigenic mouse models of focal segmental glomerulosclerosis involving pairwise interaction of CD2AP, Fyn, and synaptopodin. *J Clin Invest.* 2006; **116:** 1337-1345.

14. Löwik MM, Levtchenko EN, Monnens LA, van den Heuvel LP. WT-1 and NPHS2 mutation analysis in patients with non-familial steroid-resistant focal-segmental glomerulosclerosis. *Clin Nephrol* 2003; **59:** 143-146.

15. Schwarz K, Simons M, Reiser J *et al.* Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J Clin Invest.* 2001; **108:** 1621-1629.

16. Koziell A, Grech V, Hussain S *et al.* Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. *Hum Mol Genet* 2002; **11:** 379-388.

17. Löwik MM, Groenen PJ, Pronk I, *et al.* Focal segmental glomerulosclerosis in a patient homozygous for a CD2AP mutation. *Kidney Int* 2007; **72:** 1198-1203.

18. Liu XL, Kilpelainen P, Hellman U *et al.* Characterization of the interactions of the nephrin intracellular domain. *FEBS J.* 2005; **272:** 228-243.

19. Klamt B, Koziell A, Poulat F *et al.* Frasier syndrome is caused by defective alternative splicing of WT1 leading to an altered ratio of WT1 +/-KTS splice isoforms. *Hum Mol Genet.* 1998; **7:** 709-714.

20. Wang H, Oestreich EA, Maekawa N *et al.* Phospholipase C epsilon modulates beta-adrenergic receptor-dependent cardiac contraction and inhibits cardiac hypertrophy. *Circ Res.* 2005; **97:** 1305-1313.

21. Caridi G, Perfumo F, Ghiggeri GM. NPHS2 (Podocin) mutations in nephrotic syndrome. Clinical spectrum and fine mechanisms. *Pediatr Res* 2005; **57:** 54R-61R.

22. Hasselbacher K, Wiggins RC, Matejas V *et al.* Recessive missense mutations in LAMB2 expand the clinical spectrum of LAMB2-associated disorders. Kidney Int. 2006; **70:** 1008-1012.

23. Weber S, Gribouval O, Esquivel EL *et al.* NPHS2 mutation analysis shows genetic heterogeneity of steroid-resistant nephrotic syndrome and low post-transplant recurrence. *Kidney Int* 2004; **66:** 571-579.

24. Srivastava T, Garola RE, Kestila M *et al.* Recurrence of proteinuria following renal transplantation in congenital nephrotic syndrome of the Finnish type. *Pediatr Nephrol* 2006; **21:** 711-718.

25. Billing H, Muller D, Ruf R *et al.* NPHS2 mutation associated with recurrence of proteinuria after transplantation. *Pediatr Nephrol* 2004; **19:** 561-564.

Chapter 7

Long-term outcome of frequently relapsing minimal change nephrotic syndrome in children

Frequent relapsing and steroid dependent minimal change nephrotic syndrome (MCNS) originating in childhood can persist after puberty in over 20% of the patients. These patients require immunosuppressive treatment during several decades of their life. We examined long-term side effects of persistent nephrotic syndrome and immunosuppressive medications focussing on renal function, growth, obesity, osteoporosis, hypertension, ocular complications and fertility in adult patients with childhood-onset MCNS. Molecular analysis was performed to evaluate on a possible association of a complicated course of MCNS with podocyte gene mutations. We performed a prospective clinical examination of 15 adult patients, including serum and urine analysis, dual-energy x-ray absorptiometry (DEXA), ophthalmologic examination, semen examination and molecular analysis of *NPHS1*, *NPHS2*, *CD2AP* and *ACTN4* genes. All patients had normal glomerular filtration rate. Most frequent long-term complications were hypertension (in 8/15 patients), albuminuria (in 7/15 patients) and osteoporosis in one third of the patients. Oligospermia was found in 1 patient, reduced sperm motility in 4/8 patients and teratozoospermia in 6/8 patients. Ophthalmologic examination revealed myopia in 10/15 patients and cataract in 3/15 patients. We found *NPHS1* mutations/polymorphisms in 10/14 patients and a heterozygous *NPHS2* polymorphism in 2 patients. Children with MCNS persisting into adulthood are at risk for complications as osteoporosis, hypertension, cataract, myopia, and sperm abnormalities. Our study underscores a need for more effective and less toxic therapies for relapsing MCNS.

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Introduction

Minimal change nephrotic syndrome (MCNS) accounts for 77 % of all cases of childhood nephrotic syndrome in a series of the International Study of Kidney Diseases in children.[1] In general, long-term outcome of this disease is favourable and treatment with prednisone leads to complete remission in one third of the patients [1,2]. However, 30 % of these children develop a frequently relapsing course [1]. In this case, patients are treated with cyclophosphamide (CP). If relapses persist afterwards, treatment with cyclosporine A (CsA) is given, which allows to taper off the steroid dose, but frequently leads to CsA dependency, necessitating long-term immunosuppressive treatment. The percentage of childhood MCNS relapsing in adulthood varies from 10 to 40 % in the recent studies [2-4]. There are few data about the long-term prognosis in this group of patients, especially concerning possible side effects of the immunosuppressive medication.

While mutations in proteins expressed by glomerular podocytes were demonstrated in up to 30% of children with steroid-resistent focal segmental glomerulosclerosis (FSGS)[5,6], it remains undetermined whether underlying genetic alterations determine the susceptibility for MCNS, or predispose for a more severe course of the disease.

The aim of this study was to evaluate the long-term-outcome of children with frequently relapsing nephrotic syndrome (NS) that persisted into adulthood. We examined 15 adult patients with biopsy-proven MCNS and focussed on possible side effects of corticosteroids and cytotoxic medication such as short statue, obesity, osteoporosis, hypertension, decreased renal function and infertility. Additionally, we performed molecular analysis of four genes expressed by glomerular podocytes (*NPHS1*, *NPHS2*, *CD2AP* and *ACTN4*) to investigate whether the complicated course of MCNS was associated with mutations and/or polymorphisms in these genes.

Methods

Out of 103 patients with biopsy-proven MCNS treated in our hospital from 1971 until 2005 we identified 78 patients that were aged 16 years and older. Thirteen patients were lost to follow-up. Out of 65 patients 19 (29%) had at least one relapse of NS after puberty. Of the latter group, 15 patients with still relapsing MCNS agreed to participate in our study. The Institutional Review Board of the Radboud University Medical Centre Nijmegen approved the study. All subjects provided informed consent after explanation of the aims and methods of the study.

The clinical records of the patients were reviewed to determine the course of NS and the medications that were used. Complete remission of NS was defined as a reduction in urinary protein excretion rate to less than 4 mg/m^2/h or proteinuria less than 0.2 g/10 mmol creatinine or by 0 to trace albuminuria on dipstick during 3 consecutive days. Partial remission was defined as protein excretion between 0.2 and 2 g/10 mmol creatinine without hypoalbuminemia. A relapse-free period of minimum 2 years without immunosuppessive

medication was defined as a permanent remission. Patients were classified as frequent relapsers if they experienced 4 or more relapses in a 12 months period.

Baseline clinical and laboratory characteristics of the patients

Baseline clinical data, serum examination (urea, creatinine, glucose, HbA1C, lipids, albumin, FSH, LH, testosterone and oestradiol) and urinary analysis (albumin, creatinine, α-1-microglobulin) were obtained during complete or partial remission. Microalbuminuria was defined as urine albumin excretion between 20-300 mg/10 mmol creatinine in males and between 30-300 mg/10 mmol creatinine in females.

Short stature was defined as a height less than -2.5 SD compared to normal stature for age and sex in the Dutch population [7]. Body mass index (BMI) was calculated as weight/(height)^2 (kg/m^2). Weight excess was defined as a BMI greater than 25 in men and greater than 24 in women. Obesity was defined as a BMI greater than 30 [8]. Hypertension in adults was defined as a blood pressure of $\geq$ 140/90 mm Hg or taking medication for high blood pressure [9]. Glomerular filtration rate (GFR) was calculated as creatinine clearance by the Cockroft – Gault formula and corrected for body surface area (BSA) [10,11].

Treatment

At onset of NS all patients were treated with prednisone 60 mg/m2/day for 6-8 weeks and with prednisone 40 mg/m^2/per 2 days during subsequent 4-6 weeks. Relapses of NS were treated by prednisone 60 mg/m^2/day until the disappearance of proteinuria followed by prednisone 40 mg/ m^2/2 days for 4 weeks. Because of the frequent relapsing course of NS, CP (2-3 mg/kg/day during 8-12 weeks) was administrated in all patients after performing the renal biopsy confirming minimal change disease. All patients continued to relapse after CP course and were treated with CsA combined with steroids. The dose of CsA was regularly adapted to maintain CsA trough levels between 100-150 ng/ml and was decreased in case of suspected toxicity, which was determined as 10% rise of serum creatinine between two outpatient visits. It was attempted to decrease the dose of prednisone to the lowest possible levels for preventing relapses and long-term side effects.

Because of the relapsing disease despite of CsA treatment, further immunosuppressive therapy was determined individually as mentioned in table 1.

Ophthalmologic examination

All patients underwent a standardized ophthalmologic examination, including assessment of best-corrected visual acuity, measurement of refraction, external inspection, measurement of intraocular pressures with applanation tonometry, anterior segment slit-lamp microscopy after mydriasis of the pupils, and direct and indirect ophthalmoscopy.

Table 1 Clinical characteristics of the patients

Patiënt N	Sex	Age at onset of NS years	Age at time of study years	Number of relapses after last CP	Cum. CP dose mg/kg	Height cm	BMI kg/m²	Blood pressure mm Hg	Current medications	Duration cyclosporine therapy months
1	m	3	42	20	248	156.5	24.5	126/80	pred, CsA, ena, Ca	72
2	m	1.2	32	29	412	173.5	20.2	112/70	pred	115
3	m	6.4	17	7	154	183.5	19.8	125/80	CsA	109
4	m	2.7	28	12	176	174	22.0	130/70	pred, FK560, ena	72
5	m	2.5	36	41	414	159.5	17.6	120/75	tri, peri	66
6	m	2.2	32	9	182	183.5	31.0	120/80	pred, CP	58
7	m	3	32	10	129	184	31.4	115/70	-	7
8	m	3.6	20	34	165	175	22.2	140/70	pred, CsA, MMF	135
9	m	7.7	23	14	158	200	22.2	155/88	-	0
10	m	4.8	16	18	168	159.8	17.7	115/70	MMF	28
11	m	3	42	Unkn.	334	177	23.5	118/80	atenolol	124
12	m	5	19	26	168	169	18.8	136/64	CsA, Ca	144
13	v	2.6	18	16	150	172	19.2	110/70	pred, tacro, MMF, ena, vit D, Ca	81
14	v	7.3	23	30	Unkn.	162.5	20.5	120/80	pred, lor	148
15	v	2.6	26	>50	387	175	25.9	138/86	pred, CsA	174

Abbreviations: N, number; NS, nephrotic syndrome; CP, cyclophosphamide; Cum, cumulative; pred, prednisone; CsA, ciclosporin A; ena, enalapril; Ca, calcium; tri, triamcinolone; peri, peridopril; MMF, mycophenolate mofetil; vitD, vitamin D; lor, lorsatan; tacro, tacrolimus ; Unkn., unknown.

*Dual-energy x-ray absorptiometry (*DEXA-scan)

All patients underwent bone densitometry measurement by DEXA using a QDR 4500 densitometer (Hologic, Inc., Waltham, MA). Standard procedures supplied by the manufacturer for scanning and analyses were followed. Bone mineral content (BMC, g) and areal bone mineral density (aBMD, g/cm^2) were measured at the lumbar spine (L2–L4) and at the right femoral neck. Volumetric BMD was estimated using the formula for bone mineral apparent density (BMAD, g/cm^3): BMAD-LS (lumbar spine) = BMC/A$^{3/2}$; and BMAD-FN (femoral neck) = BMC/A^2, where A is the projected bone area. The T-score is the BMD compared to young adults expressed in standard deviation difference. T-score between –1 and –2.5 was defined as osteopenia and a T-score of less than –2.5 was defined as osteoporosis [12].

Semen analysis

Fresh semen was obtained by masturbation from 8 patients and was analysed within 2 hours. Concentration, motility and morphology were noted and WHO criteria were used for the cut-off levels [13]. The semen analysis was done once during the current study and had no defined temporal relationship to the last cyclophosphamide medication.

Molecular analysis

Genomic DNA was isolated from peripheral blood leukocytes and coding sequences of individual genes were amplified by polymerase chain reaction using intronic primers flanking the exons. Fragments included both DNA sequences of the individual exons, the splice donor and splice acceptor sites. Primer data are available on request. The following genes were screened: *NPHS1* (accession number AF190637-AF035835), *NPHS2* (AJ279246-AJ279253), *CD2AP* (AF164377-NT 007592), and *ACTN4* (NM. 004924.2). The obtained products were analyzed by double stranded DNA sequencing on a 3130 XL Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). The genomic DNA from 50 healthy controls and database search were used to confirm new mutations and exclude polymorphisms.

Results

Out of 65 adult patients with childhood-onset frequently relapsing MCNS we identified 19 patients (29%) with at least one relapse in adulthood. Clinical and laboratory data of 15 patients who agreed to participate in the study, the results of DEXA-scan and molecular analysis are presented in tables 1 and 2.

Clinical characteristics of the patients

The median follow-up period after the onset of NS was 24 years (10-39 years). The group consisted of 12 males and 3 females. The median age was 27.5 years (range 17.2-43.9 years). Median number of relapses after CP course was 18 (7 – 50). Median cumulative dose of CP was 172 mg/kg (158 – 414). Five patients had one CP course, 6 patients had a second and 3 patients also a third course of CP. The cumulative dose of steroids could not be calculated because doses given for relapse treatment could not precisely be retrieved from the patient's records. Twelve of 15 patients (80%) were still treated with immunosuppressive medication for preventing relapses of NS. The current immunosuppressive medication included prednisone (n=8), triamcinolone (n=1), CsA (n=5), tacrolimus (n=2), mycophenolate mophetil (MMF) (n=3), CP (n=1) (table 1).

Blood pressure was elevated in 2/15 patients and 6/15 patients used antihypertensive medication: ACE inhibitors (n=4), AT II antagonists (n=1) and beta blocker (n=1).
Short stature was observed in 3/12 men. The height of the female patients coped with the Dutch growth diagrams. In the male patients we observed obesity in 2 patients and a

decreased BMI (18.8 kg/m2) in one patient (table 1). No correlation was found between height, BMI and the number of relapses.

Laboratory examinations, bone densitometry and ophthalmologic examination (table2)
The GFR calculated by the Cockroft-Gault formula and corrected for BSA was normal in all patients. There were no cases of diabetes mellitus or hyperlipidaemia. No patients received cholesterol-lowering drugs.

Urinary examination showed microalbuminuria in 3/15 patients and albuminuria in 4/15 patients, while serum albumin was normal in all patients. Two patients had increased excretion of alfa-1- microglobuline (alpha-1 MG).

Osteoporosis was observed in 3/9 males and in 1/3 females. There was no correlation between BMC and BMI or height of the patients.

Ophthalmologic examination revealed myopia in 10/15 patients, bilateral posterior subcapsular cataract (PSC) in 3/15 patients, and amblyopia and convergent strabismus (esotropia) in 1/15 patients.

Fertility examination (table 3)
All patients completed normal pubertal development and achieved Tanner stages G5P5 in males and M5P5 in females. Sperm analysis was performed 0-32 years after the last CP treatment in 8 males. Hormonal status was normal in all examined men and women.

The semen analysis showed oligozoospermia in 1/8 males. Sperm motility was decreased in 4/8 subjects and 6/8 patients had teratozoospermia. Patient 6 still using CP at the time of analysis had normal sperm count and motility. Thus far one male patient fathered a child and one of the 3 females is actually pregnant.

Molecular analysis of NPHS1, NPHS2, CD2AP and ACTN4
Molecular analysis of podocyte genes demonstrated a heterozygous *NPHS1* mutation (Pro264Arg) in 3 patients, described before in patients with congenital nephrotic syndrome [14]. The known *NPHS1* Glu117Lys polymorphism was detected in a heterozygous state in 8 patients and in a homozygous state in 1 patients [15]. The *NPHS2* Arg229Gln polymorphism was found heterozygously in two patients. There was no correlation between number of relapses and the presence of genetic abnormalities. No mutations or polymorphisms leading to an amino acid substitution were detected in *CD2AP* and *ACTN4* genes.

Table 2 Results of laboratory examinations, dexa scan and molecular analysis

N	Sex	GFR	Serum examination		Urine examination		Dexa scan		Mutation analysis
		Cockroft ml/min/ 1,73m^2	Albumin g/l	Cholesterol mmol/l	Albumin mg/10 mmol creatinine	Alpha 1 MG mg/10 mmol creatinine	T-score right hip	T-score L2-L4	
1	m	131	40	4.9	861.1	7.4	-1.8	-2.3	*NPHS1* 349 G>A (E117K)
2	m	112	34	6.7	81	6.7	-1.2	-1.8	*NPHS1* 791 C>G (P264R) *NPHS2* 686 G>A (R229Q)
3	m	101	47	3.6	12.4	2.2	nd	nd	*NPHS1* 349 G>A (E117K) *NPHS1* 791 C>G (P264R)
4	m	109	44	4.8	187.6	26.2	-0.8	-2.5	*NPHS1* 349 G>A (E117K) *NPHS2* 686 G>A (R 229Q)
5	m	101	47	5.9	7	7.8	-2	-2.8	*NPHS1* 349 G>A (E117K) *NPHS1* 791 C>G (P264R)
6	m	144	39	5.7	347.6	17.7	0	-0.5	*NPHS1*ho349 G>A (E117K)
7	m	122	49	3.7	3.7	nd	nd	nd	-
8	m	131	42	5	360.3	14.3	nd	nd	*NPHS1* 349 G>A (E117K)
9	m	196	43	4.1	13.9	2.5	0	-0.8	*NPHS1* 349 G>A (E117K)
10	m	123	39	4	5.4	4.4	-2.2	-3.6	-
11	m	105	42	4	nd	10.4	-1.8	-0.9	nd
12	m	185	46	3.5	21.8	2.3	nd	nd	*NPHS1* 349 G>A (E117K)
13	v	145	29	5.2	nd	nd	-1.9	-3	*NPHS1* 349 G>A (E117K)
14	v	108	40	4.2	344.3	4.4	-0.1	-0.5	-
15	v	126	39	4	7.9	3	0.4	-0.5	-
Ref. values		> 90	35-52	4,7-6,5	males <20 females <30	< 15			

Abbreviations: N, patient number; GFR, glomerular filtration rate; Alpha1 MG, alpha-1 microglobulin; nd, not done; ho, homozygous; Ref., reference

Discussion

There are scarce data concerning the long-term outcome of children with MCNS. Two studies in the eighties conducted in the pre-cyclosporin era revealed a percentage of 5.5 % and 26 % of persistent relapses of biopsy proven MCNS in adulthood [3,4].

There are two more recent studies also including patients using calcineurin inhibitors. The first one by Fakhouri demonstrated that 42 % (43 of 102 patients) of all childhood MCNS had at least one relapse after puberty [2]. Another recent study by Ruth found that 33 % (14 of 42 patients) relapsed in adulthood [16]. Our own recent survey showed that more than 25 % of CP treated patients with childhood onset of MCNS needed medical therapy in adulthood [17]. In the latter study we found that the early age at presentation of NS

influenced the risk of frequent relapses after puberty: an age at onset under 3 years significantly correlated with a higher risk of relapses after puberty [17].

Out of 78 patients described by Kyrieleis *et al.* [17], who achieved the adult age (>16 years old), we examined 15 patients (19%) with relapsing course of NS after puberty and focused on long-term complications and possible underlying molecular defects.

Table 3 Results of fertility examination in male patients

Patient N*	Concentration 10*6/ml	Motility %	Dysmorphic head %	LH E/l	FSH E/l	Testosterone nmol/l
2	30	50	93	8.9	10.2	24
3	5	10	94	3.3	2	16
4	20	50	96	6.7	4	28
5	125	50	80	6.1	7.1	26
6	29	85	91	10.4	5.4	15
7	65	30	79	4.2	4.6	17
8	40	20	97	6	2.8	19
12	20	15	97	8.7	5.7	18
Reference values	>20	>40	<90	1.4-8.5	1.5-11	11.0-45.0

N*: patient numbers in table 3 correspond to the numbers in tables 1 and 2.

Growth

It is generally accepted that prolonged use of corticosteroids results in impaired growth [18,19]. All our patients developed nephrotic syndrome at young age and were treated with corticosteroids for years. Three of 12 male patients had impaired growth, which supports the concept that the susceptibility for corticosteroids varies highly among different patients [18]. Growth impairment was not observed in 3 female patients, however, the low number of patients precludes any conclusion. Two men also had an increased BMI > 30, indicating obesity. No correlation between the number of NS relapses and the height or BMI of the patients could be observed, admittedly the cumulative dose of the steroids could not be calculated.

Treatment

At the time of the study only 2 of 15 patients did not receive any medications and were in a permanent remission of NS. Twelve patients were still using immunosuppressive medication. Two patients had elevated blood pressure and six patients were also on antihypertensive treatment indicating that hypertension is a frequent complication in this group of patients. This is in contrast with the data of Fakouri who did not report hypertension in his patient population [2].

Renal function outcome and co-morbidity

As found in the other studies, renal function outcome was good in all patients, all of them had a normal creatinine clearance [2,16]. Our strategy of decreasing calcineurin inhibitors dose in case of serum creatinine elevation probably prevented overt renal toxicity. There were no patients with hyperlipidemia, diabetes or malignancies. Early reviews reported few cases of malignancies after protracted courses of CP, but these patients were treated continuously with CP up to 2 years [3]. It seems that the currently used dose of CP is rather safe concerning malignancies [20].

Ocular complications

Cataract and increased ocular pressure are recognized complications of prolonged steroid therapy [21]. Typically, cataracts caused by steroids are posterior subcapsular cataracts (PSC) which occur within the visual axis and therefore substantially impair visual acuity [22]. Despite its common occurrence, the mechanisms behind this complication has not been elucidated. Possibly steroids dysregulate proliferation and apoptosis of the lens epithelial cells [23,24], or can even directly bind to the lens proteins, resulting in changes of normal protein structure and lens opacities [25].

Hayasaka found in a group of 45 Japanese children with NS of various origin 33% with PSC [26]. The maximum total duration of prednisone treatment in these patients was 11 years. Nine of 45 children had a transient increase in ocular pressure during the steroid treatment, which normalized after cessation of the medication in all but one patient. Another study by Ng examined 29 children with NS (73 % with MCNS) [27]. In this study 10 % of the patients had bilateral PSC and 6.5 % - marginally elevated intraocular pressure. In our study population 20 % of the patients had PSC and no one had glaucomatous damage of the optic nerve head, possibly due to a transient character of ocular pressure elevation.

Interestingly, we observed a high prevalence of myopia, which was found in 66% of the patients. Although myopia nowadays affects over 30% of adults world-wide [28], its frequency in our patient group was twice as high. As far as we know, there are no reports on a deteriorative effect of immunosuppressive medications on ocular refraction. However, it would be important to study visual acuity and refraction in more detail in larger population of patients treated for a long time with steroids, CsA and CP. One might speculate that a transient elevation of intra-ocular pressure or changes of ion composition in the vitreous fluid due to the administration of loop diuretics could influence eye axial dimensions and refractory capacity.

Bone mineral density

Data concerning bone mineral density in patients with steroid sensitive NS are conflicting. Gulati demonstrated a decreased bone mineral content of the spine in a group of 100 children with NS in 61 % of these patients [29]. In contrast, Leonard could not show significant deficits in bone mineral content in sixty children ages 9 $\pm$3.4 years with glucocorticoid sensitive nephrotic syndrome, when correction was made for bone area, age, gender, race and Tanner stage [30,31]. Only one study dealt with adults who were treated for

childhood NS: Hegarty found in 34 adults a significantly decreased distal radial trabecular volumetric BMD (mean T-score –1.04) [32]. The authors suggest that their method is the only correct way to measure bone mineral content in patients with abnormal body size, which is often the case in subjects who have used corticosteroids [32]. Interestingly, 33/34 of the patients in this study had no relapses of NS during adult life. In contrast, all our 15 patients suffered from relapsing disease in adulthood. Therefore, the cumulative steroid dose should be higher in our group compared to the group of Hegarty. This might also explain that they found normal T-scores for BMD of the lumbar spine, whereas one third of our patients had T-scores of the lumbar spine lower than – 2.5, indicating osteoporosis.

Fertility

In the study by Hsu sperm analysis in 16 patients with idiopathic NS, 3-8 years after cytotoxic treatment, revealed azoospermia in 3/16 and oligozoospermia in 7/16 patients [33]. Six patients had normal sperm counts. The serum levels of LH and FSH were normal in most of these patients [33]. In this study there was no correlation between the cumulative dose of CP and the degree of abnormal sperm counts. However, a meta-analysis of the risk of infertility after CP treatment defined a threshold dose of 168 mg/kg [34].

We diagnosed one patient with oligozoospermia, the sperm motitlity was decreased in 4/8 subjects and the majority of patients had increased proportions of abnormal forms. The latter has been described in several studies as concurring with reduced sperm counts [17]. We did not find a correlation between the cumulative dose of CP and the risk of sperm abnormalities. The results of sperm analysis were better than expected: 7/8 patients should be able to father children according to semen quality. Female patients seem to have less risk of developing ovarial dysfunction after CP. In 12 women with NS treated by a shortcourse of CP no gonadal dysfunction was observed [35].

Molecular genetics

Alterations in podocyte genes, involved in the pathogenesis of congenital or steroid resistant nephrotic syndrome, might predispose the development of MCNS. The most frequent genetic defects found in our patient's group were heterozygous mutations or polymorphisms in the *NPHS1* gene encoding nephrin. Interestingly, these genetic defects were different from those described by Lahdenkari *et al* in a Finnish group of MCNS patients [36]. The *NPHS1* polymorphism Glu117Lys was previously described in association with decreased creatinine clearance in patients with IgA nephropathy [37]. According to the literature the allele frequency of this polymorphism is 36.7% in an ethnically matched control population.[15] A similar allele frequency of 33.3% was found in our group of patients. The previously described polymorphism in *NPHS2* (Arg229Gln), associated with increased risk of microalbuminuria [38], was detected on one allele of two MCNS patients. Tsukaguchi described an allele frequency of 3.6% for this polymorphism [39]. Since *in vitro* translated Arg229Gln podocin showed a decreased binding efficiency to nephrin, the possibility that *NPHS2* Arg229Gln enhances susceptibility to focal segmental glomerulosclerosis in association with a second mutant *NPHS2* allele is suggested [39]. In our study population the

allele frequency for *NPHS2* Arg229Gln was only slightly higher (6.7%). Because of comparable frequency of genetic alterations in NPHS1 and NPHS2 genes between our patient's group and healthy population, it seems unlikely that these defects make podocytes more susceptible for nephrotic insults.

In conclusion, at least one quarter of patients with childhood-onset frequently relapsing MCNS will continue to relapse in adult life and require prolonged medical therapy. Although these patients maintain normal GFR, they frequently develop extra-renal complications such as osteoporosis, hypertension and decreased visual acuity due do cataracts and myopia. One third of male patients treated with CP demonstrate decreased sperm count and motility. These findings indicated that new more effective and less toxic therapies should be searched for relapsing MCNS.

References

1. Tarshish P, Tobin JN, Bernstein J, Edelman CM. Prognostic significance of the early course of minimal change nephrotic syndrome: report of the International Study of Kidney Disease in Children. *J Am Soc Nephrol.* 1997; **8:** 769-776.

2. Fakhouri F, Bocquet N, Taupin P *et al.* Steroid-sensitive nephrotic syndrome: From childhood to adulthood. *Am J Kidney Dis.* 2003; **41:** 550-557.

3. Trompeter RS, Lloyd BW, Hicks J *et al.* Long-term outcome for children with minimal–change nephrotic syndrome. *Lancet.* 1985; **1:** 368-370.

4. Lewis MA, Baildom EM, Davis N *et al.* Nephrotic syndrome: From toddlers to Twenties. *Lancet.* 1989; **1:** 255-259.

5. Woroniecki RP, Kopp JB. Genetics of focal segmental glomerulosclerosis. *Pediatr Nephrol.* 2007; **22:** 638-644.

6. Löwik M, Levtchenko E, Westra D *et al.* Bigenic heterozygosity and the development of steroid-resistant focal segmentalglomerulosclerosis. *Nephrol Dial Transplant.* 2008; **23:** 3146-3151.

7. Fredriks AM, van Buuren S, Verloove-Vanhorick SP. Handleiding groeidiagrammen 2004. In Handleiding groeidiagrammen 2004 1st ed., edited by Bohn Stafleu van Loghum 2004.

8. WHO. Physical status : the use and interpretation of anthropometry. Report of a WHO Expert Committee. WHO Technical Report Series 854: World Health Organisation 1995.

9. Kidney Disease Outcomes Quality Initiative (K/DOQI). K/DOQI clinical practice guidelines on hypertension and antihypertensive agents in chronic kidney disease. *Am J Kidney Dis.* 2004; **43:** 1-290.

10. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron.* 1976; **16:** 31-35.

11. DuBois D, DuBois EF. A formula to estimate the approximate surface area if height and weight be known. *Arch Intern Medicine.* 1916; **17:** 863-871.

12. Pols HAP, Wittenberg J. CBO-richtlijn 'Osteoporose' (tweede herziening). *Ned Tijdschr Geneeskd.* 2002; **146:** 1359-1363.

13. World Health Organization: WHO Laboratory manual for the examination of human semen and sperm–cervical mucus interaction, 4th ed., edited by Cambridge University Press, Cambridge, 1999.

14. Beltcheva O, Martin P, Lenkkeri U, Tryggvason K. Mutation spectrum in the nephrin gene (NPHS1) in congenital nephrotic syndrome. *Hum Mutat.* 2001; **17:** 368-373.

15. Lenkkeri U, Männikkö M, McCready P *et al.* Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations. *Am J Hum Genet.* 1999; **64:** 51-61.

16. Ruth EM, Kemper MJ, Leuman EP *et al.* Children with steroid-sensitive nephrotic syndrome come of age: Long term outcome. *J Pediatr.* 2005; **147:** 202-207.

17. Kyrieleis HA, Levtchenko EN, Wetzels JF. Long-term outcome after cyclophosphamide treatment in children with steroid-dependent and frequently relapsing minimal change nephrotic syndrome. *Am J Kidney Dis.* 2007; **49:** 592-597.

18. Emma F, Sesto A, Rizzoni G. Long-term linear growth of children with severe steroid-responsive nephrotic syndrome. *Pediatr Nephrol.* 2003; **18:** 783-788.

19. Donatti TL, Koch VH, Fujimura MD, Okay Y. Growth in steroid-responsive nephrotic syndrome: a study of 85 pediatric patients. *Pediatr Nephrol.* 2003; **18:** 789-795.

20. Latta K, von Schnakenburg C, Ehrich JHH. A meta-analysis of cytotoxic treatment for frequently relapsing nephrotic syndrome in children. *Pediatr Nephrol.* 2001; **16:** 271-282.

21. Carnahan MC, Goldstein DA. Ocular complications of topical, peri-ocular, and systemic corticosteroids. *Curr Opin Ophthalmol.* 2000; **11:** 478-483.

22. James ER. The etiology of steroid cataract. *J Ocul Pharmacol Ther.* 2007; **23:** 403-420.

23. Petersen A, Carlsson T, Karlsson JO *et al.* Effects of dexamethasone on human lens epithelial cells in culture. *Mol Vis.* 2008; **14:** 1344-1345.

24. Gupta V, Awasthi N, Wagner BJ. Specific activation of the glucocorticoid receptor and modulation of signal transduction pathways in human lens epithelial cells. *Invest Ophthalmol Vis Sci.* 2007; **48:** 1724-1734.

25. Bucala R, Manabe S, Urban RC, Cerami A. Nonenzymatic modification of lens crystallins by prednisolone indu ces sulfhydryl oxidation and aggregate formation: in vitro and in vivo studies. *Exp Eye Res.* 1985; **41:** 353-363.

26. Hayasaka Y, Hayasaka S, Matsukura H. Ocular findings in Japanese children with receiving prolonged corticosteroid therapy. *Ophthalmologica.* 2006; **220:** 181-185.

27. Ng JS, Wong W, Law RW *et al.* Ocular complications of paediatric patients with nephrotic syndrome. *Clin Experiment Ophthalmol.* 2001; **29:** 239-243.

28. Crewther SG, Murphy MJ, Crewther DP. Potassium channel and NKCC cotransporter involvement in ocular refractive control mechanisms. *PLoS ONE.* 2008; **3:** e2839.

29. Gulati S, Sharma RK, Gulati K *et al.* Longitudinal follow-up of bone mineral density in children with nephrotic syndrome and the role of calcium and vitamin D supplements. *Nephrol Dial Transplant.* 2005; **20:** 1598-1603.

30. Leonard MB. Glucocorticoid-induced osteoporosis in children: impact of the underlying disease. *Pediatrics.* 2007; **119 Suppl 2:** 166-174.

31. Leonard MB, Feldman HI, Shults J *et al.* Long-term, high-dose glucocorticoids and bone mineral content in childhood glucocorticoid-sensitive nephrotic syndrome. *N Engl J Med.* 2004; **351:** 868-875.

32. Hegarty J, Mughal MZ, Adams J, Webb NJA. Reduced bone mineral density in adults treated with high-dose corticosteroids for childhood nephrotic syndrome. *Kidney Int.* 2005; **68:** 2304-2309.

33. Hsu AC, Folami AO, Bain J, Rance CP. Gonadal function in males treated with cyclophosphamide for nephrotic syndrome. *Fertil Steril.* 1979; **31:** 173-177.

34. Wetzels JFM. Cyclophosphamide-induced gonadaltoxicity: a treatment dilemma in patients with lupus nephritis. *Neth J Med.* 2004; **62:** 347-352.

35. Bogdanovic R, Banicevic M, Cvoric A. Pituitary-gonadal function in women cyclophosphamide treatment for childhood nephrotic syndrome: long-term follow-upstudy. *Pediatr Nephrol.* 1990; **4:** 455-458.

36. Lahdenkari AT, Kestilä M, Holmberg C *et al.* Nephrin gene (NPHS1) in patients with minimal change nephrotic syndrome (MCNS). *Kidney Int.* 2004; **65:** 1856-1863.

37. Narita I, Goto S, Saito N *et al.* Genetic polymorphism of NPHS1 modifies the clinical manifestations of Ig Anephropathy. *Lab Invest.* 2003; **83:** 1193-1200.

38. Pereira AC, Pereira AB, Mota GF *et al.* NPHS2 R229Q functional variant is associated with microalbuminuria in the general population. *Kidney Int.* 2004; **65:** 1026-1030.

39. Tsukaguchi H, Sudhakar A, Le TC *et al. NPHS2* mutations in late-onset focal segmental glomerulosclerosis: R229Q is a common disease-associated allele. *J Clin Invest.* 2002; **110:** 1659–1666.

Chapter 8

A glomerulus-specific agrin knockout mouse – Practical Information

It is a general consideration that the permeability of the glomerular capillary wall is based on both size and charge of the proteins. In the glomerular basement membrane (GBM) the heparan sulphate proteoglycans (HSPG's) are responsible for the anionic charge of this structure. Agrin is the major HSPG of the GBM and believed to play a considerable role in the charge-dependent permeability of the glomerular capillary wall. Agrin knockout mice die at birth with severe neuromuscular defects. To investigate the precise role of agrin in charge-dependent permeability, the aim was to develop a glomerulus-specific agrin knockout mouse model. The strategy was to generate a mutant mouse with floxed agrin genes. By subsequent crossbreeding with mice expressing cre-recombinase under the control of a nephrin-promoter, a mutant mouse deficient of agrin in the glomerulus only would be generated. Embryonic stem cells with a f-lox P flanked agrin gene were created by homologous recombination and were used to develop the mouse with floxed agrin genes. Unfortunately, germline transmission was not successful, thereby preventing the generation of the conditional mouse model. Recent studies in this field and new insights in the role of agrin in the charge-dependent permeability are discussed.

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Introduction

The filtration of the glomerular capillary wall is based on size- and charge-dependent permeability. The glomerular filtration barrier consists of a fenestrated endothelium covered by an anionic glycocalyx, the glomerular basement membrane (GBM) existing of extracellular matrix proteins and acting like a sieve, and finally the visceral epithelial cells or podocytes with cytoplasmic extensions called foot processes also covered by a glycocalyx. The structure of the glomerular filtration barrier determines the size-dependent permeability, while certain specific components are important for the permeability based on charge. Both endothelial cells and podocytes have an anionic glycocalyx, largely formed by glycosaminoglycan (GAG) sidechain containing proteoglycans and podocalyxin respectively [1,2]. Morphological alterations in the endothelial glycocalyx have functional consequences for glomerular permeability [2,3]. Injection of GAG-degrading enzyme chondroitinase in an experimental mouse model resulted in a decreased thickness of the endothelial cell glycocalyx and in an increase of albumin clearance [3]. A decreased thickness of the glycocalyx resulting in an increased flux of albumin is also seen when conditionally immortalized human glomerular endothelial cells are treated with neuraminidase, heparinise III, and human heparinitase II [2]. Podocalyxin is important in maintaining podocyte foot process structure. Podocalyxin deficient (-/-) mice exhibit profound defects in kidney development because podocytes fail to form foot processes and slit diaphragms [4]. These mice die within 24 hours after birth with anuric renal failure [4].

It is generally believed that the GBM is of primary importance in charge-dependent permeability. The anionic charge of the GBM is caused by proteoglycans. Proteoglycans consist of a core protein with Glycosaminoglycan (GAG) sidechains, mainly heparan sulphate GAG (HSPG's) [5,6]. Three different HSPG's have been identified in basement membranes so far: perlecan and collagen XVIII are mainly present in the mesangial matrix and Bowman's capsule [7,8], whereas agrin is predominantly found in the GBM [9,10]. The agrin core protein carries at least two GAG chains (figure 1a) [10], mostly HS but also chondroitin sulphate (CS) [11].

Several studies have shown the importance of heparan sulphate (HS) for the charge-dependent permeability. In animal models, enzymatic removal or neutralization of GBM-HS results in proteinuria [6,12,13]. Human and experimental glomerulopathies show a reduced amount of GBM-HS [14,15] and a reduced staining for GBM-HS is correlated with an increase of albuminuria in both human glomerulopathies and animal models [16-18].

HSPG mutant mouse models show different effects on the function and structure of the glomerulus, dependent on the core proteins that are affected by homologous recombination. Mice lacking the perlecan HS binding sites show no kidney malfunctions or proteinuria but have small eyes with degenerating lenses and show a greater susceptibility of protein-overload proteinuria [19,20]. In these mice the mutant perlecan compensates the HS loss with attachment of other GAG chains like CS [20]. Ocular abnormalities are also seen in mice lacking collagen XVIII (Col18a1 -/-) [21]. The collagen XVIII deficiency also leads to structurally altered basement membranes and hydrocephalus in some cases [22]. In the

glomerulus mild mesangial expansion is seen and also elevated serum creatinine levels were measured [22]. The authors did not look if proteinuria was present in these mice. The effect of agrin deficiency in mice is more dramatic. These mice have a normal development, but die at birth with sever neuromuscular defects [23]. For this reason, we tried to develop a glomerulus-specific agrin knockout mouse to analyse the role of this major HSPG in the function of the glomerular ultrafiltration. This chapter describes the strategy used to achieve this goal. Unfortunately, due to lack of germ line transmission, it was not possible to generate glomerulus-specific agrin knockout mice. Finally, we will discuss literature about this subject published after our attempt to develop a glomerulus-specific agrin knockout mouse.

Materials and Methods

Construction of the floxed agrin targeting vector and introduction in Embryonic Stem cells.
The targeting vector was constructed using genomic DNA from an Ola129 mouse strain. The arms of homology were amplified by PCR and inserted into a pBluescript SKII (Stratagene) already containing a phosphoglycerine kinase (PGK)-Hygromycin selection cassette flanked by Lox P sites (pBS-Hygro). Three arms of homology are inserted (figure 1b) using unique restriction sites. The left arm (L) constitutes exon 2 through a part of intron 5 and is approximately 1950 bp. This arm is inserted in pBs-Hygro at the 5' site of the selection cassette. The middle arm (M) of ~1200 bp continues through intron 9 and is placed at the 3' site of the floxed (Lox P flanked) Hygromicin selection cassette. At the 3' end of the middle arm, an additional Lox P site was added. To assure a proper insertion of this Lox P site into the mouse genome through homologous recombination, a right arm (R ~4710 bp) was inserted directly after the Lox P site and this arm starts in intron 9 and exceeds until exon 22. The vector was linearized with NotI and 15-20 µg was electroporated in embryonic stem (ES) cells (~$50*10^6$) derived from an Ola129 mouse strain. Electroporation conditions: 250 Volt and 500 µF. Putative homologous recombinants were isolated after ten days selection with hygromycin (300 µg/ml) and DNA was isolated for Southern Blot analysis.

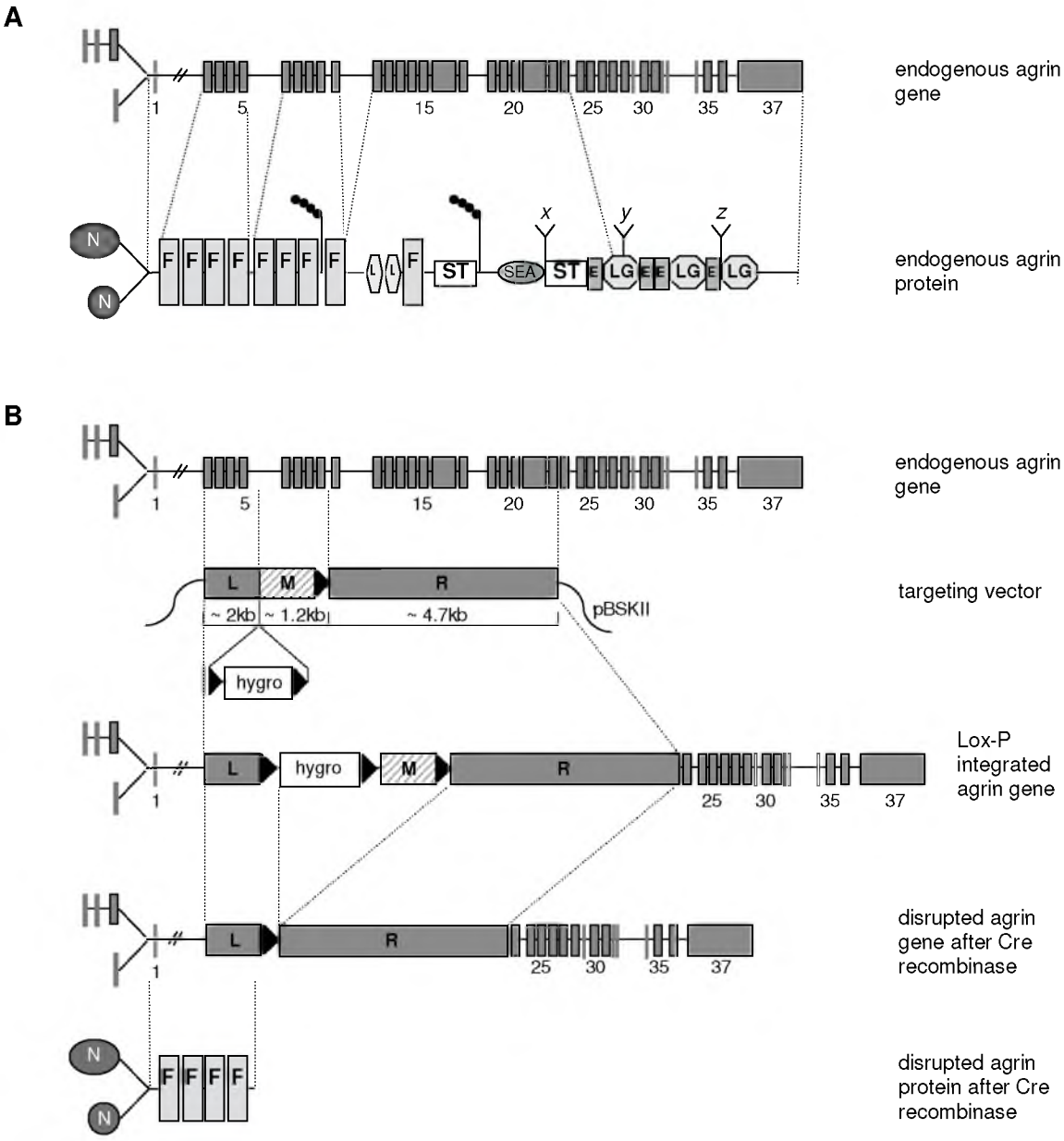

Figure 1 Gene strategy glomerulus-specific agrin knockout mouse. **A.** The endogenous agrin gene with intron/exon boundaries. The filled boxes represent the exons and are numbered starting from the first shared exon after the distinct N-termini. Underneath, the structure of endogenous agrin with distinct N-terminal domains (N), alternative splice sites x, y and z, and two putative GAG attachment sites. F = follistatin-like repeats, L= laminin EGF like, S/T = serine/threonine rich, SEA = sperm protein/enterokinase/agrin, E = EGF like, LG = laminin globular domain. **B.** The targeting vector for the floxed agrin mouse model. Three arms of homology (L = exon 2/intron 5, M = intron 5/intron 9, and R = intron 9/exon 22) are inserted in pBSKII-Hygro. An additional Lox P site (triangle) was inserted between arm M and R in intron 9. Homologous recombination between the homology arms L, M, R and the endogenous agrin gene leads to the insertion of the Lox P flanked hygromycin cassette in intron 5 and the insertion of the third Lox P site in intron 9 (Lox P integrated agrin gene). Glomerulus-specific agrin knockout mice are generated by crossbreeding the floxed agrin mice with mice expressing cre-recombinase under the control of a nephrin-promoter. Cre-recombinase disrupts the agrin gene by removing exon 6 to 9 (disrupted agrin gene after Cre recombinase), resulting in a

frame shift directly after exon 5 and a premature stop-codon 33 bp after the shift. The disrupted agrin protein only contains the first four follistatin-like repeats (disrupted agrin protein after Cre recombinase).

Molecular Analysis

DNA was digested with *HindIII* and separated on a 0.8% agarose gel. For Southern Blotting, the gel was denaturated for 1 hour in 1.5M NaCl / 0.5M NaOH and neutralized for 1 hour in 1M TrisHCl / 1.5M NaCl at room temperature. The DNA was transferred O/N on nitrocellulose paper (Millipore) by capillary action in 2xSSC and then baked for 2 hours at 80°C. Blots were prehybridised in 6xSSC, 0.5% SDS, 5x Denhardt's solution and 100 µg/ml denaturated Salmon testis DNA for 2-4 hours at 68°C. For the hybridization additional 0.01M EDTA and ^{33}P-labeled probe were added. Probes were made by PCR using complement primers and ^{33}P-labeled dCTP. Blots were hybridized O/N at 68°C. After washing the blot in 2xSSC / 0.5% SDS the bands were visualized using X-ray film.

Positive clones are karyotyped to establish the number of chromosomes in the clones. ES cells were cultured in 6-wells plates and at 80% confluency, Colcemid (200 ng) was added to the media and cells were incubated for 1 hour at 37°C. After trypsinizing and pelleting, three ml KCl (75mM, 37°C) was added to resuspend the cells (10 min at 37°C). Cells were washed three times with fixative (Methanol:Glacial Acetic acid = 3:1) and after the final wash resuspended in ~10 drops of fixative. The cell-suspension was dropped on a microsope slide and stained with Giemsa (10%). Further analysis of Southern-blot-positive clones was done by PCR.

ES Injection and Breeding

The ES cells were injected into blastocysts isolated from C57Bl6J mice according to standard protocol at MURINUS GmbH, Hamburg, Germany.

Results

Targeting Vector

In order to achieve a kidney specific agrin knock-out mouse, a targeting vector (figure 1b) is used to produce mice with floxed agrin genes. Crossbreeding these mice with mice expressing cre-recombinase under the control of a nephrin promoter [24], will result in glomerulus-specific agrin deficient mice. Three arms of homology were successfully inserted into pBs-Hygro. The strategy using three arms of homology instead of the normal two, was performed in order to achieve a frameshift resulting in a premature stop codon after cre-recombinase treatment. Detailed study of the structure of the agrin gene showed us this is only possible if the Lox P sites are placed in intron 5 and 9. The third most right arm was placed in the targeting vector to ensure proper insertion of the Lox P site in intron 9 by homologous recombination. After homologous recombination, the targeting vector will insert a floxed hygromycin cassette in intron 5 of the agrin gene and, as stated before, an

additional Lox P site in intron 9. Thus, the mutated gene would lack exon 6 through exon 9 after cre-recombinase. This results in a premature stop-codon 11 amino acids directly after exon 5. The linearized vector was electroporated in embryonic stem (ES) cells derived from Ola129 mice. Hygromycin selection resulted in the formation of clones. These clones were isolated and grown further for DNA isolation.

Molecular Analysis

To assure proper homologous recombination, Southern Blot analysis was performed. For Southern Blot analysis, genomic DNA from hygromycin selected clones and wildtype DNA were digested with *HindIII* and probed with ^{33}P-labeled probes. Two probes (figure 2a) were used which both give a band of 10.1 kb in wildtype DNA. Positive clones also give a band of 4 and 4.8 kb for probe 1 and 2 respectively. Of the clones isolated only two were found positive for both probes (figure 2b).

Positive clones were further analysed by PCR. To further exclude random integration of the targeting vector into the genome of the ES cell, we performed a PCR using a forward primer situated in exon 21 and a reverse primer in the pBlue vector. Visualisation of a band of 1197 bp indicates random integration, while no band implies a successful homologous recombination. Figure 2b (left panel) shows a band for randomly integrated vector DNA (-) while one of the positive clones (+) revealed no band.

A second PCR was performed to test the presence of the third Lox P site in intron 9. The forward primer in exon 9 and reverse primer in exon 10 give rise to a band of 280 bp in wildtype DNA and a band of 370 bp when Lox P is inserted. Figure 2b (right panel) clearly shows two bands of both the wildtype allele and the mutated allele with the additional Lox P site for the positive clone (+). Both PCR's clearly confirm the results found with the Southern Blot. Both clones were found positive for homologous recombination.

To exclude any chromosomal degeneration, a karyotype was performed for both clones. The result of one clone is shown in figure 2c. The karyogram shows a normal number of 40 chromosomes.

ES Injection and Breeding

Positive ES cells were injected into E3.5 blastocysts isolated from C57Bl6J mice and replaced in C57Bl6J foster mothers. Six litters were born. Many pups were small and died shortly after birth. One litter was destroyed by the mother. Six chimeric mice, recognizable by their striped fur colour, survived. Three of them were male and first used to crossbreed with wildtype mice in order to get mice heterozygous for the floxed agrin gene. Since several attempts were unsuccessful, also the female chimeric mice were crossbred with wildtype mice. Also these did not lead to heterozygous mice with floxed agrin gene. Therefore, it was not possible to undertake the additional steps to generate glomerulus-specific agrin knockout mice. The designed glomerulus-specific agrin knockout mice would express an aberrant agrin protein, as Cre-recombinase excision would result in a disrupted agrin gene with removal of exon 6 to 9 which consequently results in an agrin protein that only contains the first four follistatin-like repeats.

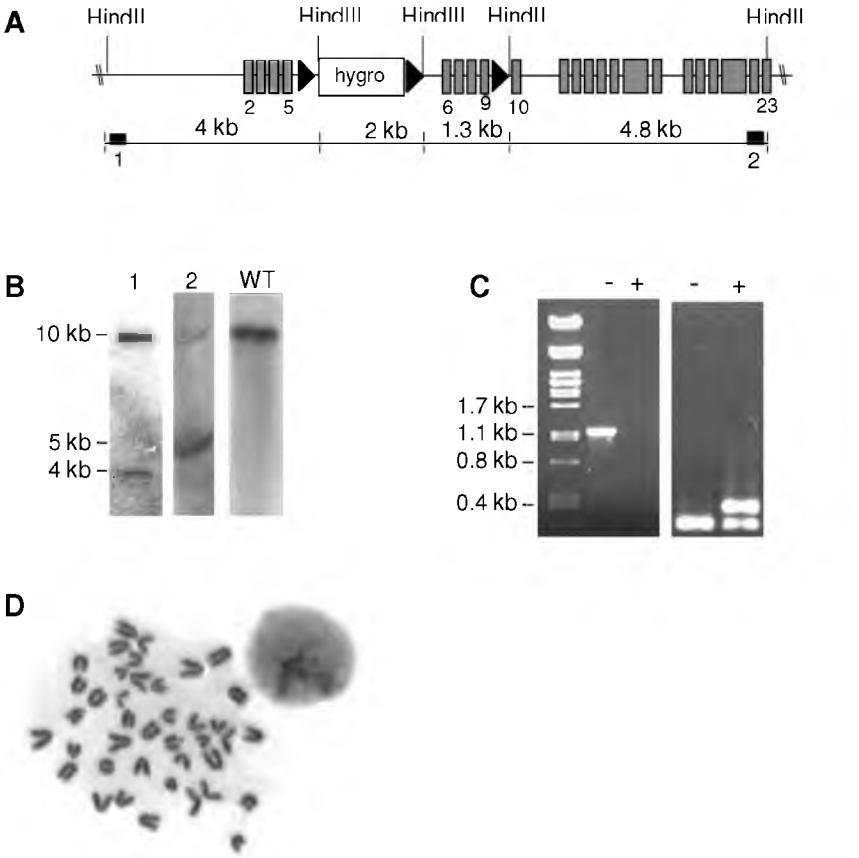

Figure 2 Molecular analysis of hygromycin selected ES clones. **A.** Floxed agrin gene after homologous recombination. Restriction enzyme *HindIII* is used for Southern Blotting. The *HindIII* restriction sites labelled with an asterisks are native sites and result in a band of 10.1 kb using probe 1 or 2 (filled boxes numbered 1 or 2) when no Lox P sites are integrated (the so-called WT allele). The additional *HindIII* sites give a band of 4 and 4.8 kb for probes 1 and 2 respectively in case of homologous recombination. **B.** Northern Blot analysis of *HindIII* digested DNA. Wildtype DNA (lane marked WT) shows one band of 10.1 kb. Probe 1 and 2 (lanes marked 1 and 2) show an additional band of 4 and 4.8 kb respectively in positive clones. **C.** PCR analysis of positive clones. Random integration of the targeting vector is seen in a negative clone (left panel, lane marked -) since the PCR with a forward primer situated in exon 21 and a reverse primer situated in pBlueScript shows a band of approximately 1.2 kb. No band is seen in the positive clone after homologous recombination (lane marked +). The right panel shows a positive clone with two bands representing wildtype intron 9 (~280 bp) and the mutated Lox P containing intron 9 (~370 bp), while the negative clone only shows the wildtype band. **D.** Karyogram of a positive clone with 40 chromosomes.

Discussion

Agrin is the major heparan sulphate proteoglycan in the glomerular basement membrane and a decrease in heparan sulphate is generally associated with proteinuria. To investigate the precise role of agrin in the glomerular filtration, we tried to develop a glomerulus-specific agrin knockout mouse. This chapter describes the steps taken to achieve this goal. Unfortunately, we were not able to develop this mouse model because of the lack of germline transmission of the f-lox P mutated agrin allele.

The results described in this chapter shows a successful set up of the gene strategy necessary to develop the glomerulus-specific agrin knockout mouse. To ensure the targeting vector would result in a frameshift and a premature stop of the agrin protein, Lox P sites were placed in intron 5 and 9. For this reason, the targeting vector had to have three arms of homology. This gives rise to several ways of homologous recombination when transformed in ES cells. Homologous recombination could take place between the left and middle arm, the left and right arm, and the middle and right arm, with the latter not being viable because of the hygromycin selection. Because of the two possibilities left, more clones had to be checked for proper homologous recombination. Furthermore, since no negative selection cassette (like thymidine kinase (TK)) was used for random integration of the targeting vector, the number of false positive clones was even higher. Nevertheless we succeeded to find two positive clones as proven by Southern Blot and PCR analysis.

Very important in the development of transgenic animals when using ES cells, is the condition of these cells. They need to be of low passage number to lower the change of differentiation of the cells. Pluripotency is also more guaranteed when a differentiation-inhibition factor like LIF (Leukemia Inhibitory Factor) is added to the medium during culturing. In addition, culturing the cells on a layer of “feeder” cells (irradiated or mytomycin treated mouse embryonic fibroblasts) ensures pluripotency since feeder cells also produce LIF but are not able to divide any more. Non-pluripotent ES cells are not suitable for the generation of transgenic mice because this lowers the change of germline transmission dramatically. The quality of our clones used to develop glomerulus-specific agrin knockout mice was apparently not sufficient for germline transmission. Most likely this can be attributed to the pluripotency of the used ES cells, since another attempt to generate mutant mice using the same batch of ES cells, failed as well.

Recently, glomerulus-specific agrin deficient mice were developed [25]. The gene targeting resulted in a frameshift (deletion exon 7 until 34) and truncated agrin proteins that lack most of the C terminus. Mice homozygous for the deficiency had no phenotypic effect. N-terminal fragments of agrin (deletion from the first follistatin-like domain on) were synthesized and properly localized to the GBM. The truncated N-terminal fragments were not glycanated, neither did any other GBM-HSPG compensate for the loss of HS. The anionic charge of the GBM was dramatically reduced but glomerular ultrastructure was indistinguishable and no pathological abnormalities were observed. These mice show that agrin is not essential for the initial assembly of the glomerular capillary wall or for its maintenance. In all fairness, it is possible that the still synthesized N-terminal fragments of

agrin in the mutant mice have retained unknown functions to ensure proper incorporation and assembly of the GBM [25].

Another *in vivo* study also shows no major role for HS in the permselectivity of the glomerulus [26]. Injection of heparinase III into rats resulted in removal of HS without alterations in expression levels of the agrin core-protein. This cleavage of HS did not result in proteinuria in these rats [26].

Why is it that former studies in animal models and human glomerulopathies show an important role for HS in glomerular permeability [25-27]?. *In vivo* perfusion of heparinase in rats resulted in an increase of native ferritin permeability [6], but other GAG-degrading enzymes did not [27]. Moreover, in this study no thorough check was performed whether HS was removed completely [26]. Loss of HS in human glomerulopathies like membranous nephritis, lupus nephritis, and diabetic nephropathy has been observed [11,12] as well as in animal models [13]. However, a recent study has shown no change in heparan sulphate structure in early human and experimental diabetic nephropathy indicating that the loss of HS might be a secondary event in a more advanced stage of the disease [28].

The studies discussed in the previous paragraph will become a subject of debate whether or not the GBM and/or agrin is involved in the charge-dependent permeability of the glomerular capillary wall or if this role is more attributed to the glycocalyx of the endothelium and/or visceral epithelial cells. Future studies will give more clearance in this.

References

1. Kerjaschki D, Sharkey DJ, Farquhar MG. Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell. *J Cell Biol.* 1984; **98:** 1591-1596.

2. Singh A, Satchell SC, Neal CR *et al.* Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. *J Am Soc Nephrol.* 2007; **18:** 2885-2893.

3. Jeansson M, Haraldsson B. Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier. *Am J Physiol Renal Physiol.* 2006; **290:** F111-116.

4. Doyonnas R, Kershaw DB, Duhme C *et al.* Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin. *J Exp Med.* 2001; **194:** 13-27.

5. Kanwar YS, Farquhar MG. Anionic sites in the glomerular basement membrane. In vivo and in vitro localization to the laminae rarae by cationic probes. *J Cell Biol.* 1979; **81:** 137-153.

6. Kanwar YS, Linker A, Farquhar MG. Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *J Cell Biol.* 1980; **86:** 688-693.

7. Groffen AJ, Hop FW, Tryggvason K *et al.* Evidence for the existence of multiple heparan sulfate proteoglycans in the human glomerular basement membrane and mesangial matrix. *Eur J Biochem.* 1997; **247:** 175-182.

8. Saarela J, Rehn M, Oikarinen A *et al.* The short and long forms of type XVIII collagen show clear tissue specificities in their expression and location in basement membrane zones in humans. *Am J Pathol.* 1998; **153:** 611-626.

9. Groffen AJ, Ruegg MA, Dijkman H *et al.* Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. *J Histochem Cytochem.* 1998; **46:** 19-27.

10. Groffen AJ, Buskens CA, van Kuppevelt TH *et al.* Primary structure and high expression of human agrin in basement membranes of adult lung and kidney. *Eur J Biochem.* 1998; **254:** 123-128.

11. Winzen U, Cole GJ, Halfter W. Agrin is a chimeric proteoglycan with the attachment sites for heparan sulfate/chondroitin sulfate located in two multiple serine-glycine clusters. *J Biol Chem.* 2003; **278:** 30106-30114.

12. Rosenzweig LJ, Kanwar YS. Removal of sulfated (heparan sulfate) or nonsulfated (hyaluronic acid) glycosaminoglycans results in increased permeability of the glomerular basement membrane to 125I-bovine serum albumin. *Lab Invest.* 1982; **47:** 177-184.

13. van den Born J, van den Heuvel LP, Bakker MA *et al.* A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats. *Kidney Int.* 1992; **41:** 115-123.

14. Wada N, Ueda Y, Iidaka K *et al.* Portions of basement membrane with decreased negative charge in various glomerulonephritis. *Clin Nephrol.* 1990; **34:** 9-16.

15. Washizawa K, Kasai S, Mori T *et al.* Ultrastructural alteration of glomerular anionic sites in nephrotic patients. *Pediatr Nephrol.* 1993; **7:** 1-5.

16. van den Born J, van den Heuvel LP, Bakker MA *et al.* Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases. *Kidney Int.* 1993; **43:** 454-463.

17. Tamsma JT, van den Born J, Bruijn JA *et al.* Expression of glomerular extracellular matrix components in human diabetic nephropathy: decrease of heparan sulphate in the glomerular basement membrane. *Diabetologia.* 1994; **37:** 313-320.

18. van Bruggen MC, Kramers K, Hylkema MN *et al.* Decrease of heparan sulfate staining in the glomerular basement membrane in murine lupus nephritis. *Am J Pathol.* 1995; **146:** 753-763.

19. Rossi M, Morita H, Sormunen R *et al.* Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. *EMBO J.* 2003; **22:** 236-245.

20. Morita H, Yoshimura A, Inui K *et al.* Heparan sulfate of perlecan is involved in glomerular filtration. *J Am Soc Nephrol.* 2005; **16:** 1703-1710.

21. Ylikarppa R, Eklund L, Sormunen R *et al.* Lack of type XVIII collagen results in anterior ocular defects. *FASEB J.* 2003; **17:** 2257-2259.

22. Utriainen A, Sormunen R, Kettunen M *et al.* Structurally altered basement membranes and hydrocephalus in a type XVIII collagen deficient mouse line. *Hum Mol Genet.* 2004; **13:** 2089-2099.

23. Gautam M, Noakes PG, Moscoso L *et al.* Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell.* 1996; **85:** 525-535.

24. Eremina V, Wong MA, Cui S, Schwartz L, Quaggin SE. Glomerular-specific gene excision in vivo. *J Am Soc Nephrol.* 2002; **13:** 788-793.

25. Harvey SJ, Jarad G, Cunningham J *et al.* Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity. *Am J Pathol.* 2007; **171:** 139-152.

26. Wijnhoven TJ, Lensen JF, Wismans RG *et al.* In vivo degradation of heparan sulfates in the glomerular basement membrane does not result in proteinuria. *J Am Soc Nephrol.* 2007; **18:** 823-382.

27. Harvey SJ, Miner JH. Breaking down the barrier: evidence against a role for heparan sulfate in glomerular permselectivity. *J Am Soc Nephrol.* 2007; **18:** 672-674.

28. van den Born J, Pisa B, Bakker MA *et al.* No change in glomerular heparan sulfate structure in early human and experimental diabetic nephropathy. *J Biol Chem.* 2006; **281:** 29606-29613.

Chapter 9

Summary, general discussion, and future perspectives

Podocytes are highly specialized, terminally differentiated epithelial cells with cytoplasmic extensions, called foot processes, involved in the glomerular ultrafiltration. Adjacent foot processes form slit pores with a width of approximately 25-40 nm. These slit pores are covered with a membrane and many researchers have investigated the structure of this slit membrane as being the leading part of the glomerular filtration barrier. Although the structure is not completely elucidated yet, several data from animal models and/or genetic analyses suggest a substantial role of slit membrane proteins, and proteins involved in maintaining the slit membrane structure, in the development of nephrotic syndrome. Other aspects of the glomerular filtration barrier are not discussed here [1].

The objective of the studies described in this thesis was to determine to what extent podocyte genes are involved in the development of congenital nephrotic syndrome, and to provide insight in the role of these genes in the development of focal segmental glomerulosclerosis (FSGS), diffuse mesangial sclerosis (DMS) and minimal change nephrotic syndrome (MCNS).

The involvement of podocyte genes in the development of nephrotic syndrome - and focal segmental glomerulosclerosis

To investigate the involvement of podocyte genes in the development of childhood onset steroid-resistant non-familial FSGS, we tested 20 patients for mutations in seven podocyte genes (*NPHS1, NPHS2, CD2AP, WT1, ACTN4, TRPC6* and *PLCE1*) and for the mtDNA A3243G transition generally involved in the MELAS syndrome (chapter 2, 5, and 6). No mutations were found in the *ACTN4* and *TRPC6* genes and no patient was found to have the A3243G transition. In four patients, mutations were found which could explain the development of the nephropathy. They are discussed in the next four paragraphs. Of these four patients, two had an additional heterozygous *NPHS1* mutation. The role of these additional heterozygous *NPHS1* mutations is unknown.

Bigenic heterozygosity: NPHS2 and CD2AP

Single heterozygous mutations in genes normally involved in a recessive mode of inherited FSGS (like *NPHS2*), are frequently reported. The significance of these heterozygous mutations is not clear so far, but may be explained in several ways. Other yet unidentified mutations in the regulatory or intronic sequence may be present. The heterozygous mutations may also lead to an enhanced susceptibility to develop proteinuria triggered by environmental factors as seen in mouse models. Depending on the genetic background, heterozygous *NPHS2* knockout mice develop proteinuria when aging [2], and heterozygous *CD2AP* knockout mice are more susceptible to glomerular injury by nephrotoxic antibodies and immunocomplexes [3]. Finally, multigenic inheritance may cause the development of nephrotic syndrome. This statement was investigated in mouse models. CD2AP$^{+/-}$ mice were crossbred with either Synaptopodin$^{+/-}$ or Fyn proto-oncogene$^{+/-}$ mice. Synaptopodin and Fyn proto-oncogene have an interaction with CD2AP. All three heterozygous mice had a relatively unremarkable clinical phenotype. Bigenic heterozygosity, however, resulted in an increased incidence of proteinuria and pathological changes resembling FSGS [4].

A role of bigenic heterozygosity in the development of FSGS is also seen in our patient group (chapter 2 and 6). One patient presented with a maternally inherited heterozygous *NPHS2* mutation and a heterozygous paternally inherited *CD2AP* mutation. Both parents had normal creatinine levels and no proteinuria. Podocin (*NPHS2*) oligomerization clusters nephrin at the slit diaphragm and CD2AP serves as an adapter protein to the overall structure. Bigenic heterozygosity in genes closely involved in the slit membrane assembly may result in an aberrant assembly and ultimately proteinuria and nephrotic syndrome. The precise role of bigenic heterozygosity in the pathophysiology of FSGS needs to be elucidated in further studies.

Homozygous CD2AP mutation

Recently, a heterozygous *CD2AP* splice site mutation, affecting 2 nucleotides, was found by Kim *et al* [3] in two patients with primary FSGS. The mutation results in aberrant splicing leading to truncation of 80% of the protein. Immunoblot analysis of CD2AP expression from lysates of immortalized B lymphocytes from the patients showed a lower CD2AP expression [3].

In our rather small group of twenty patients, two were found to have *CD2AP* mutations, suggesting an important role of this protein in the development of FSGS. Besides the bigenic heterozygous case mentioned in the previous paragraph, we also found a homozygous *CD2AP* mutation in a patient from consanguineous parents (chapter 5). The R612Stop mutation affects the COOH-terminus of CD2AP and the premature stopcodon would result in a 4% truncated protein. The mutation is situated in the F-actin interacting domain and *in vitro* studies showed a reduced binding efficiency of the mutated CD2AP with F-actin. Immunostaining lymphocytes of the patient with CD2AP antibody, demonstrated a lack of CD2AP expression in the patient, while both parents, who are heterozygous for the mutation, had a (close to) normal CD2AP value. In mouse models, homozygous CD2AP knockout mice show proteinuria, already in week 2, and die around 6-7 weeks [5]. The phenotype of heterozygous CD2AP knockout mice is less severe [3]. These mice show no proteinuria, but develop glomerular lesions consisting of mild to extensive mesangial expansion and occasionally immunoglobulin deposits at age 9 months [3]. The *CD2AP* splice site mutation found in the two primary FSGS patients was also of heterozygous state. In case of our patient, the parents, who are heterozygous for a *CD2AP* nonsense mutation, show no proteinuria. The difference between the outcome of both heterozygous *CD2AP* mutations may be explained by the severity of the truncation, but it is possible that in the patients with the splice site mutation, a second hit in another gene is present (bigenic heterozygosity). In these patients, no other podocyte genes were analyzed. Recently three heterozygous mutations were reported by Gigante *et al* [6] in a study of 80 Italian patients with idiopathic steroid resistant nephrotic syndrome

Compound heterozygous NPHS2 mutations

Hereditary steroid resistant nephrotic syndrome is frequently associated with mutations in the podocyte gene podocin, *NPHS2*. In our group, renal disease in one patient could be explained by a compound heterozygous podocin mutation (chapter 6).

The knowledge of having podocin mutations is important for determining treatment modalities. Aggressive treatment with immunosuppressive drugs can be avoided and the favorite prognosis of the renal transplant can be more or less predicted. Early studies showed no recurrence of proteinuria after renal transplantation in patients having a *NPHS2* mutation. However, later on, several patients did show recurrence of proteinuria, and it is now believed that the finding of a *NPHS2* mutation does not definitely exclude the recurrence of nephrotic syndrome in the renal graft. Collective data from patients with *NPHS2* mutations who received a renal transplant showed an unexplained higher risk for recurrence in patients having a heterozygous mutation, compared to patients with homozygous or compound

heterozygous mutations [2]. This group of patients should be closely monitored in the post-graft phase.

De novo WT1 mutation

In the group of twenty patients with non-familial steroid-resistant FSGS, we found one mutation in the *WT1* gene (chapter 2), frequently found in patients with the Frasier syndrome (FS).

Denys-Drash syndrome (DDS) and FS are closely related syndromes characterized by nephrotic syndrome (due to DMS (DDS) or FSGS (FS)), male pseudohermaphroditism and a higher risk of developing Wilm's tumor (DDS) or gonadoblastoma/dysgerminoma (FS). Both DDS and FS are caused by *de novo* mutations in the transcription factor WT1. Proteomic investigation of glomerular podocytes from a DDS patient indicated a role of WT1 in the regulation of expression of key components and regulators of the cytoskeleton [7].

The patient with the *WT1* mutation has a normal female phenotype and a XX karyotype. Our finding of a *WT1* gene mutation has practical implications for the patient as regular control of gonads and kidneys is required because of the risk of gonadoblastoma and, more rarely in FS, Wilms tumours.

The mtDNA A3243G transition in adult FSGS patients

No A3243G transition in the mtDNA, associated with the MELAS syndrome, was found in the group of childhood-onset FSGS patients. mtDNA is present in heteroplasmic state with mutated and wildtype DNA coexisting in cells. Since the amount of mutated mtDNA has to exceed a certain threshold value to cause a phenotype, and this is more likely to happen while aging, we also analyzed a group of adult-aged steroid-resistant FSGS patients (chapter 3).

In one patient the A3243G transition was found (30% in blood sample and 68% in urine sample). This patient developed full-blown nephrotic syndrome during pregnancy, which persisted after delivery. Steroid treatment was complicated by the development of diabetes mellitus, and also her sister had in the meantime developed insulin-dependent diabetes mellitus. The sister has the A3243G transition in a lower percentage; 25% in blood sample and 28% in urine sample. At the time of DNA analyses, the sister had no signs of renal disease. Electron microscopy of the patient showed podocyte foot process effacement and abnormal mitochondria.

The mtDNA A3245G transition can be associated with FSGS and severe nephrotic syndrome. Clinicians should be aware of the clinical heterogeneity of mitochondrial cytopathies, and should be suspicious of patients with steroid-resistant FSGS who present with some particular features such as deafness, diabetes, neuromuscular symptoms, cardiomyopathy or a family history positive for any of these.

Conclusion

FSGS may be subdivided into three categories: idiopathic, genetic, and secondary due to injury, medication, or drug abuse. We investigated to what extent the genetic factor plays a

role in the development of FSGS in patients with childhood onset steroid-resistant FSGS. With the number of mutations found, we would like to conclude that podocyte genes play a substantial role in the development of FSGS, and moreover, the role of multigenic inherited podocyte gene mutations may be substantial in this as well. For this reason, and because the knowledge of having a genetic defect is important for future treatment modalities, it is important to provide a standard molecular analysis assay for these patients.

The involvement of podocyte genes in the development of nephrotic syndrome - and diffuse mesangial sclerosis

De novo mutations in the transcription factor *WT1* are frequently found in patients with DDS and DMS. We screened patients with DMS for the presence of a *WT1* mutation and found in one patient with inherited nephrotic syndrome and a dysgerminoma in the right abdomen at age 17 years, a mutation confirming the diagnosis DDS (chapter 4).

The *WT1* mutation had far-reaching consequences for the further treatment and follow-up of the patient. A XY-karyotype was established in this patient with female genitalia, and the second gonad was removed because of the higher risk of malignant degeneration. The rudimentary kidneys were removed since the risk of developing Wilms' tumour. Both kidneys showed nephroblastosis, also a benign abnormality and possibly a pre-stage of Wilms tumour.

This case shows the importance of *WT1* mutation analysis in patients with DMS and informs physicians responsible for the health of adults about previously untreatable genetic diseases of childhood in order to provide adequate medical management of these patients.

Not much is known about the genetic role in the development of DMS. Mutations in the *WT1* gene are frequently found in complete or in incomplete forms of DDS, but do not explain cases of DMS without genital abnormalities or malignancies. Recently, mutations in the *PLCE1* gene (phospholipase C epsilon) and *LAMB2* gene (laminin β2) were found in patients with familial form of DMS. *PLCE1* mutations were found in patients with early onset nephrotic syndrome and rapid progression to end stage renal disease (ESRD) [8]. Notably, two individuals responded to therapy. *LAMB2* mutations are associated with the Pierson syndrome (microcoria-congenital nephrotic syndrome with early progression to ESRD), but are also found in patients with congenital nephrotic syndrome without eye anomalies or with minor ocular changes [9]. The exact role of these genes in the development of DMS needs to be elucidated in future studies.

The involvement of podocyte genes in the development of minimal change nephrotic syndrome

To investigate the role of podocyte genes in the development of MCNS, we screened 15 MCNS patients with frequently relapsing course of the disease, persisting after puberty, for

mutations in the genes *NPHS1, NPHS2, CD2AP, WT1*, and *ACTN4* (chapter 7). We did not find homozygous or compound heterozygous mutations in these genes which could explain the pathology. However, two heterozygous *NPHS1* mutations (P264R in 3 patients, and T1152M in 1 patient), and one known *NPHS1* polymorphism (E117K, heterozygous in 1 patient and homozygous in 2) were found. This polymorphism is associated with decreased creatinine clearance in patients with IgA nephropathy [10]. Also one heterozygous *NPHS2* polymorphism (R229Q) associated with microalbuminuria in the general population was found in one patient. These results support the statement that genetic changes in nephrin may have a pathogenic role in some MCNS patients [11]. The genetic changes might make the glomerular slit membrane more vulnerable for a 'second hit' causing proteinuria and MCNS.

No mutations were found in *NPHS2*, *CD2AP*, *WT1*, and *ACTN4* suggesting no role for these genes in the pathogenity of MCNS. However, a *WT1* splice site mutation in a 46XX female patient with MCNS and Wilms' tumor has been reported [12]. One report also describes *NPHS2* mutations in MCNS patients; R138Q (heterozygous in one family and homozygous in one family and two sporadic cases) and 855-856delAA (homozygous in one sporadic case). In the family with heterozygous R138Q mutation, two members had steroid-resistant nephrotic syndrome. Remarkably, in one patient the renal biopsy revealed MCNS, while the other family member was diagnosed with FSGS [13]. Recently a lowered expression of galectin-1, normally binding to nephrin, is reported in MCNS [14].

Future perspectives

The common denominator of all FSGS variants is a podocyte disease. Increased knowledge, originating from inherited podocyte disease, will direct the future treatment. New hereditary disorders will be discovered and elucidate new functions of the podocyte. Careful clinical studies are needed to establish genotype/phenotype correlations. When possible, podocytes should be obtained of these patients. The development of conditionally immortalized human, and mouse, podocyte cell lines, have considerably advanced the field. All aspects of podocyte biology can be approached [15]. It makes micro-array and proteomics technology possible [7] and RNA interference can be applied [16]. The technique for obtaining human podocytes in culture from urine should be vigorously continued [17].

Experimental work in animals also contributes to the progress in this field, especially since it is possible to restrict or delete expression to the podocyte. Specific podocyte depletion causing glomerulosclerosis was reached by diphtheria-toxin injections in rats expressing the human diphtheria-toxin receptor under the control of a podocin promoter [18]. Ablation of developing podocytes disrupts cellular interactions and nephrogenesis both inside and outside the glomerulus [19]. Animal models, however, do not always reflect the human disease. PLCE-1 knockout mice do not have a nephrosis-like phenotype [8]. In the zebrafish, using antisense morpholino oligonucleotides to knockdown the PLCE-1 ortholog, the glomerular barrier function was disturbed [8]. It may be interesting to use the zebrafish more

frequently in the future. Another useful model may be the in Drosophila described podocyte-like cell type the nephrocyte. It closely resembles the mammalian podocyte, including the nephrin-based slit diaphragm. Endothelial cells, however, are lacking in the filtration barrier [20].

When exposed to nephrotic plasma samples, nephrin, podocin and CD2AP assumed a cytoplasmic distribution in cultured human podocytes, and nephrin and synaptopodin were selectively downregulated. Nephrin could be relocated to the plasma membrane by co-incubation with non-nephrotic plasma samples, indicating that nephrotic plasma seems to be deficient in factors that act via the podocyte slit membrane complex [21]. This factor, or these factors, may be lost in urine [22]. The glomerular albumin permeability significantly reduces when isolated rat glomeruli are incubated with serum and an equal volume of urine of patients with a nephrotic syndrome. This is not seen when the nephrotic serum is mixed with normal control urine [22].

Podocytes are not isolated cells. VEGF produced by the podocytes binds to VEGF receptors on endothelial cells, inducing endothelial fenestration and maintaining glomerular permeability. Anti-VEGF therapy may induce glomerular endothelial swelling with loss of endothelial fenestrae and effacement of foot processes. Thrombotic microangiopathy has been reported [23]. Angiopoetin I originating from the podocyte may stabilize the endothelium and resist angiogenesis [24].

In the concept of Meyrier [25], FSGS starts with podocyte injury which drives the glomerular lesions to build up of fibrosis and scarring. Besides being an inherited podocyte disease, FSGS may also be due to an immunological disorder, a viral cause or to a toxic compound [26]. Corticoids and Cyclosporin remain the mainstay of therapy based on the concept that the lesions and proteinuria are due to an immunological assault [27,28]. However, the beneficial effect of Cyclosporin on proteinuria turns out not to be caused by inhibition of signaling of activated T cells, but rather results from the stabilization of the actin cytoskeleton in kidney podocytes by prevention of synaptopodin degradation [29]. Also, in the *in vitro* model of puromycin-aminonucleoside induced podocyte injury, glucocorticoids protect and enhance recovery via actin filament stabilization [30]. Angiotensin II has not only a dynamic effect on the renal circulation, but also induces a reorganization of F-actin fibers and a redistribution of ZO-1 in murine podocytes accompanied by increased albumin permeability [31].

Mature podocytes are postmitotic cells that do not proliferate. The podocytes, however, can be regenerated from a resident population of renal progenitors localized within the parietal epithelium of Bowman's capsule mainly at the urinary pole. Injection of these cells into mice with adriamycin-induced nephropathy reduces proteinuria and improved glomerular damage [32]. These data illustrate that by studying the biology of the podocyte, a new therapy can emerge or at least better understood.

References

1. Salmon AHJ, Neal CR, Harper SJ. New aspects of glomerular filtration barrier structure and function: five layers (at least) not three. *Curr Opin Nephrol Hypertens*. 2009; **18:** 197-205.

2. Caridi G, Perfumo F, Ghiggeri GM. NPHS2 (Podocin) mutations in nephrotic syndrome. Clinical spectrum and fine mechanisms. *Pediatr Res*. 2005; **57:** 54R-61R.

3. Kim JM, Wu H, Green G *et al*. CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. *Science*. 2003; **300:** 1298-1300.

4. Huber TB, Kwoh C, Wu H *et al*. Bigenic mouse models of focal segmental glomerulosclerosis involving pairwise interaction of CD2AP, Fyn, and synaptopodin. *J Clin Invest*. 2006; **116:** 1337-1345.

5. Shih NY, Li J, Karpitskii V, Nguyen A *et al*. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science*. 1999; **286:** 312-315.

6. Gigante M, Pontrelli P, Montemurno E *et al*. CD2AP mutations are associated with sporadic nephrotic syndrome and focal glomerulosclerosis (FSGS). *Nephrol Dial Transplant*. 2009; **24:** 1858-1864.

7. Viney RL, Morrison A, van den Heuvel *et al*. A proteomic investigation of glomerular podocytes from a Denys-Drash syndrome patient with a mutation in the Wilms tumour suppressor WT1. *Proteomics* 2007; **7:** 804-815.

8. Hinkes B, Wiggins RC, Gbadegesin R *et al*. Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. *Nat Genet*. 2006; **38:** 1397-1405.

9. Hasselbacher K, Wiggins RC, Matejas V *et al*. Recessive missense mutations in LAMB2 expand the clinical spectrum of LAMB2-associated disorders. *Kidney Int*. 2006; **70:** 1008-1012.

10. Narita I, Goto S, Saito N *et al*. Genetic polymorphism of NPHS1 modifies the clinical manifestations of Ig A nephropathy. *Lab Invest*. 2003; **83:** 1193-1200.

11. Lahdenkari AT, Kestila M, Holmberg C, Koskimies O, Jalanko H. Nephrin gene (NPHS1) in patients with minimal change nephrotic syndrome (MCNS). *Kidney Int*. 2004; **65:** 1856-1863.

12. Loirat C, Andre JL, Champigneulle J *et al*. WT1 splice site mutation in a 46,XX female with minimal-change nephrotic syndrome and Wilms' tumour. *Nephrol Dial Transplant*. 2003; **18:** 823-825.

13. Karle SM, Uetz B, Ronner V *et al*. Novel mutations in NPHS2 detected in both familial and sporadic steroid-resistant nephrotic syndrome. *J Am Soc Nephrol*. 2002; **13:** 388-393.

14. Shimizu M, Khoshnoodi J, Akimoto Y *et al*. Expression of galectin-1, a new component of slit diaphragm, is altered in minimal change nephrotic syndrome. *Lab Invest* 2009; **89:** 178-195.

15. Shankland SJ, Pippin JW, Reiser J, Mundel P. Podocytes in culture: past, present, and future. *Kidney Int*. 2007; **72:** 26-36.

16. Fan Q, Xing Y, Ding J *et al*. The relationship among nephrin, podocin, CD2AP, and alpha-actinin might not be a true 'interaction' in podocyte. *Kidney Int*. 2006; **69:** 1207-1215.

17. Vogelmann SU, Nelson WJ, Myers BD, Lemley KV. Urinary excretion of viable podocytes in health and renal disease. *Am J Physiol Renal Physiol*. 2003; **285:** F40-48.

18. Wharram BL, Goyal M, Wiggins JE *et al*. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *J Am Soc Nephrol*. 2005; **16:** 2941-2952.

19. Jia Q, Bradley W, McDill *et al*. Ablation of developing podocytes disrupts cellular interactions and nephrogenesis both inside and outside the glomerulus. *Am J Physiol Renal Physiol* 2008; **295:** F1790-1798.

20. Simons M, Huber TB. Flying podocytes. *Kidney Int* 2009; **75:** 455-457.

21. Coward RJ, Foster RR, Patton D *et al.* Nephrotic plasma alters slit diaphragm-dependent signaling and translocates nephrin, podocin, and CD2 associated protein in cultured human podocytes. *J Am Soc Nephrol.* 2005; **16:** 629-637.

22. Carraro M, Zennaro C, Candiano G *et al.* Nephrotic urine prevents increased rat glomerular albumin permeability induced by serum from the same patient with idiopathic nephrotic syndrome. *Nephrol Dial Transplant.* 2003; **18:** 689-693.

23. Eremina V, Jefferson A, Kowalewska J *et al.* VEGF inhibition and renal thrombotic microangiopathy. *N Engl J Med* 2008; **358:** 1129-1136.

24. Satchell SC, Anderson KL, Mathieson PW. Angiopoietin I and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties. *J Am Soc Nephrol* 2004; **15:** 566-574.

25. Meyrier A. Mechanisms of disease: focal segmental glomerulosclerosis. *Nat Clin Pract Nephrol.* 2005; **1:** 44-54.

26. Meyrier A. Nephrotic focal segmental glomerulosclerosis in 2004: an update. *Nephrol Dial Transplant.* 2004; **19:** 2437-2444.

27. Barisoni L, Schnaper W, Kopp JB. Advances in the biology and genetics of the podocytopathies-implications for diagnosis and therapy. *Arch Pathol Lab Med* 2009; **133:** 201-216.

28. Marasa M, Kopp JB. Monoclonal antibodies for podocytopathies: rationale and clinical responses. *Nat Rev Nephrol* 2009; **5:** 337-348.

29. Faul C, Donnelly M, Merscher-Gomez S *et al.* The actin cytoskeleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A. *Nat Med.* 2008; **14:** 931-938.

30. Ransom RF, Lam NG, Hallett MA *et al.* Glucocorticoids protect and enhance recovery of cultured murine podocytes via actin filament stabilization. *Kidney Int.* 2005; **68:** 2473-2483.

31. Macconi D, Abbate M, Morigi M *et al.* Permselective dysfunction of podocyte-podocyte contact upon angiotensin II unravels the molecular target for renoprotective intervention. *Am J Pathol.* 2006; **168:** 1073-1085.

32. Ronconi E, Sagrinati C, Angelotti ML *et al.* Regeneration of glomerular podocytes by human renal progenitors. *J Am Soc Nephrol* 2009; **20:** 322-332.

Nederlandse Samenvatting

Achtergrond en doelstelling van het onderzoek

Het glomerulaire filtratie systeem bestaat uit tenminste drie lagen. De filtratie laag bestaande uit podocyt cellen speelt een belangrijke rol bij de ontwikkeling van het nefrotisch syndroom. Patiënten met een nefrotisch syndroom verliezen belangrijke en nuttige bestanddelen, zoals eiwitten, via de urine (proteïnurie).

Podocyt cellen zijn enorm gespecialiseerde cellen met uitlopers die " foot processes" worden genoemd. Deze "foot processes" zijn onderling met elkaar verbonden door een dun membraan, het " slit membraan". De laatste jaren is veel duidelijk geworden over de samenstelling en opbouw van dit "slit membraan". Wanneer de samenstelling van het 'slit membraan" om welke reden dan ook afwijkend is, kan dit tot het nefrotisch syndroom leiden.

Het hier beschreven onderzoek houdt zich bezig met podocyt eiwitten die betrokken zijn bij het ontstaan van een nefrotisch syndroom, dat morfologisch gekenmerkt wordt door focale segmentale glomerulosclerose (FSGS). Bij FSGS ontstaat de proteinurie doordat in een aantal, maar niet alle (focaal) glomeruli bindweefsel is gevormd en bovendien is slechts een deel van de glomerulus aangedaan (segmentaal). FSGS is een histopathologische bevinding en geen ziektebeeld op zich. Het heeft daarom verschillende achterliggende oorzaken, die onder andere genetisch kunnen zijn. Om deze reden hebben we in een groep patiënten met FSGS de genen coderend voor podocytaire eiwitten, betrokken bij de opbouw van het slit membraan, in detail onderzocht. Deze genen zijn ook geanalyseerd bij patiënten met diffuus mesangiale sclerose (DMS) en patiënten met minimale laesies (MCNS) om de rol hiervan bij de ontwikkeling van deze ziektes te bepalen.

Podocyt genen spelen een belangrijke rol in de ontwikkeling van FSGS

Een groep van 20 FSGS patiënten (FSGS ontstaan in de kinderleeftijd) is geselecteerd op basis van het niet reageren op de standaard behandeling met steroïden en bij deze patiënten is er niet sprake van een familiale vorm van FSGS. Bij deze groep is een mutatie analyse uitgevoerd in een aantal podocyt genen, te weten *NPHS1,* coderend voor nefrine, *NPHS2,* coderend voor podocine*, CD2AP*, *WT1*, *ACTN4*, *TRPC6* en *PLCE1*. Daarnaast is gekeken naar de mitochondriële DNA (mtDNA) afwijking A3243G welke over het algemeen geassocieerd wordt met het MELAS syndroom (myopathie, encefalopathie, lactaatacidose, 'stroke- like' episoden), maar ook gevonden wordt bij patiënten met een minder progressieve vorm van FSGS. Geen mutaties werden gevonden in de *ACTN4*, *TRPC6* en *PLCE1* genen, evenmin de A3243G mutatie in mtDNA. In 4 patiënten werden mutaties gevonden in *NPHS2*, *CD2AP* en *WT1* die het ontstaan van FSGS zouden kunnen verklaren omdat mag worden verondersteld/is aangetoond dat door de mutaties in deze genen de vorm en/of de functie van het gecodeerde eiwit in de slit-membraan veranderd is.

Hoofdstuk 2 beschrijft een patiënt met FSGS waarin een *WT1* mutatie is gevonden. De transcriptie factor WT1 is betrokken bij het ontstaan van het Denys-Drash (DDS) en Frasier (FS) syndroom, twee sterk op elkaar lijkende syndromen gekenmerkt door een

nefrotisch syndroom, mannelijke pseudohermafroditisme en het ontwikkelen van maligniteiten. Het kennisnemen van deze mutatie is belangrijk voor de verdere behandeling van de patiënt, aangezien WT1 mutanten een verhoogd risico hebben op het ontwikkelen van maligniteiten.

Erfelijke steroïd-resistente nefrotisch syndroom wordt over het algemeen geassocieerd met mutaties in het podocine (*NPHS2*) gen. Bij één patiënt kon de FSGS pathologie verklaard worden door een compound heterozygote mutatie in dit gen (hoofdstuk 6). Ook voor mutaties in het *NPHS2* gen, evenals *WT1*, geldt dat het kennisnemen van een mutatie in dit gen belangrijke gevolgen heeft voor de verdere behandeling van de patiënt. Onderzoek heeft laten zien dat bij aanwezigheid van een mutatie in *NPHS2* verdere agressieve behandeling met steroïden, cyclophosphamide en cyclosporine vermeden kan worden en de prognose na niertransplantatie min of meer voorspeld kan worden. Aanvankelijk werd verondersteld dat bij een patiënt met een podocine mutatie op een van de allelen (heterozygoot), de kans op proteinurie na transplantatie van de patiënt sterk werd gereduceerd. Inmiddels is door het nauwkeurig vastleggen van gegevens het inzicht gewijzigd en wordt verondersteld dat patiënten met een enkele heterozygote mutatie juist meer risico lopen op terugkeer van proteïnurie dan patiënten met een homozygote of compound heterozygote mutatie.

Regelmatig worden enkele heterozygote mutaties gevonden in genen die normaal gesproken betrokken zijn bij een autosomale recessieve vorm van FSGS (zoals bv *NPHS2*). Onlangs is in muismodellen aangetoond dat heterozygotie in twee genen kan leiden tot een nefrotisch syndroom. Muizen heterozygoot knockout voor *CD2AP* zijn gekruist met muizen heterozygoot knockout voor synaptopodine (eiwit betrokken bij de opbouw van het cytoskelet van de podocyt) of Fyn (enzym dat andere podocyt-eiwitten fosforyleert). Deze muizen hebben geen afwijkende fenotype, maar de nakomelingen laten proteïnurie en FSGS zien. Ook in onze groep patiënten is bij één patiënt twee heterozygote mutaties gevonden (hoofdstuk 2 en 6) in twee verschillende genen: *NPHS2* (afkomstig van de moeder) en *CD2AP* (afkomstig van de vader). Beide ouders hebben geen proteïnurie en normale serum creatinine waarden. Heterozygotie in meerdere genen coderend voor eiwitten betrokken bij de opbouw van het slit membraan, kan dus ook in de mens leiden tot een afwijking in de opbouw en uiteindelijk proteïnurie en nefrotisch syndroom.

Een mutatie in het *CD2AP* gen is slechts één keer beschreven in de literatuur. Onze groep patiënten laat twee mutaties zien in dit gen en dit suggereert een grotere rol van dit gen bij de ontwikkeling van FSGS. Hoofdstuk 5 beschrijft een nieuwe CD2AP mutatie in een patiënt met steroïd-resistente FSGS. De mutatie (1834 C>T, R612Stop) resulteert in een vroegtijdige stopcodon en wordt in de patiënt homozygoot gevonden. Beide (gerelateerde) ouders hebben de mutatie op een van de allelelen (zijn heterozygoot), maar hebben geen afwijkende nierfunctie. Onze biochemische studie laat zien dat het gemuteerde eiwit in mindere mate aan actine bindt. Bovendien is er geen CD2AP expressie waargenomen in geïsoleerde lymfocyten van de patiënt, terwijl de ouders een normale hoeveelheid laten zien ten opzichte van controle lymfocyten. We concluderen dat heterozygote CD2AP mutaties niet perse tot nierfalen leiden.

Mitochondriële aandoeningen zijn erg heterogeen van aard vanwege de eigenschappen van het mtDNA. mtDNA wordt maternaal overgeërfd en is, in tegenstelling tot kern-DNA, ongelijk verdeeld over de cellen. Bovendien komen in de cellen wildtype kopieën van het mtDNA voor naast gemuteerd mtDNA (heteroplasmie). Om tot een bepaald ziektebeeld te leiden, moet de hoeveelheid gemuteerd mtDNA een bepaalde drempelwaarde overstijgen. De kans dat dit gebeurt neemt toe in leeftijd van de patiënt. Om die reden hebben we naast de groep patiënten waarbij de FSGS op kinderleeftijd ontstond, ook gekeken naar volwassen patiënten met steroïd-resistente FSGS en het voorkomen van de A3243G mutatie in het mtDNA. In hoofdstuk 3 wordt een patiënt beschreven met de A3243G mutatie. In tegenstelling tot voorheen beschreven gevallen, waarbij minder progressieve vormen van FSGS beschreven werden, ontwikkelde deze patiënt een uitgesproken nefrotisch syndroom gedurende haar zwangerschap met resistentie voor steroïden wat voort duurde na de bevalling. Een zus van de patiënt had diabetes mellitus ontwikkeld. Ons advies is dat artsen mitochondriële aandoeningen in overweging zouden moeten nemen wanneer doofheid, diabetes mellitus of neuromusculaire aandoeningen aanwezig zijn bij de patiënt of familieleden.

De rol van podocyt genen bij de ontwikkeling van DMS

Niet veel is bekend over de rol van podocyt genen bij de ontwikkeling van DMS. Mutaties in het *WT1* gen, wordt vaak gevonden bij patiënten met DMS in relatie tot de complete of in-complete vorm van DDS, maar verklaart niet de geïsoleerde gevallen van DMS. Zeer recentelijk zijn er mutaties beschreven in de *PLCE1* en *LAMB2* genen bij patiënten met DMS. De precieze rol van deze en eventueel andere genen bij de ontwikkeling van DMS zal in de toekomst onderzocht worden.

Zoals eerder aangeduid heeft het vinden van een *WT1* mutatie gevolgen voor de verdere behandeling van de patiënt, waarbij tevens psychische problemen kunnen ontstaan. Hoofdstuk 4 beschrijft de casus van een 17-jarige vrouw met een afwezige seksuele ontwikkeling en een nefrotisch syndroom vanaf de geboorte dat heeft geleid tot nierfalen. Bij haar werd de diagnose ‘Denys-Drash-syndroom’ (DDS) gesteld nadat zich bij haar een dysgerminoom van een ovarium had ontwikkeld en een *WT1* mutatie was vastgesteld. De tweede gonade evenals de rudimentaire natieve nieren werden verwijderd in verband met het verhoogde risico op het ontwikkelen van maligniteiten. Tevens werd een XY-karyotype bij haar vastgesteld. Artsen verantwoordelijk voor de behandeling van volwassenen worden steeds vaker geconfronteerd met de gevolgen van ziekten ontstaan op kinderleeftijd. Deze casus geeft de noodzaak weer deze artsen kennis te laten nemen van voorheen onbehandelbare erfelijke kinderziekten om zo een adequate behandeling van deze patiënten te kunnen garanderen.

Een bescheiden rol van podocyte genen in de ontwikkeling van MCNS

Om de rol van podocyte genen in de ontwikkeling van MCNS te onderzoeken hebben we in een groep van 15 patiënten gekeken naar mutaties in de genen *NPHS1*, *NPHS2*, *CD2AP*, *WT1* en *ACTN4* (hoofdstuk 7). Geen mutaties werden gevonden die direct de pathologie zouden kunnen verklaren. Wel werd een heterozygote *NPHS2* polymorfisme (R229Q) gevonden die in een normale populatie tot microalbuminurie kan leiden. Ook twee heterozygote *NPHS1* mutaties en een bekende *NPHS1* polymorfisme (E117K) werden gevonden. Deze laatste polymorfisme wordt geassocieerd met een verlaagd creatinine klaring bij patiënten met IgA nefropathie. Deze resultaten ondersteunen de gedachte dat genetische veranderingen in het nefrine gen een rol speelt in MCNS. Deze verandering maakt het glomerulaire slit membraan waarschijnlijk kwetsbaar voor een 'second hit'.

Toekomstperspectieven

Nieuwe erfelijke aandoeningen zullen in de toekomst ontdekt worden. Zorgvuldig uitgevoerde klinische studies zullen de relatie tussen genotype en fenotype vaststellen. Indien mogelijk, zouden specifiek de podocyten geïsoleerd kunnen worden uit bijvoorbeeld urine-monsters van de patiënten. De ontwikkeling van geïmmortaliseerde humane, en muis, podocyt cellijnen heeft namelijk het onderzoeksveld een grote impuls gegeven. Deze ontwikkeling maakt technieken als micro-array en proteomics mogelijk als mede RNA interferentie. Optimalisatie van de procedure voor isolatie van de podocyten uit urinemonsters is nog noodzakelijk.

Een belangrijke rol in het onderzoek spelen ook de diermodellen, met name sinds het mogelijk is bepaalde genen weefselspecifiek te beïnvloeden. Echter, diermodellen geven niet altijd de humane pathologie weer, zoals te zien is bij *PLCE1* knockout muizen. Deze muizen laten geen nefrotisch ziektebeeld zien. In de zebravis wordt deze pathologie wel gevonden als de PLCE1 homoloog uitgeschakeld wordt. Om de rol van agrine in het glomerulair filtratiesysteem te onderzoeken, hebben wij geprobeerd een glomerulus-specifieke agrine knockout muis te ontwikkelen. Hoofdstuk 8 beschrijft de wijze waarop wij geprobeerd hebben dit doel te bereiken. Helaas zijn we er niet in geslaagd het diermodel te ontwikkelen, omdat er geen kiembaan transmissie heeft plaatsgevonden.

Toekomstig onderzoek zal ook proberen een antwoord te vinden op de vraag welke factor of welke factoren missen in het serum van patiënten met een nefrotisch syndroom. Wanneer podocyt cellen blootgesteld worden aan serum van deze patiënten, vindt er relocatie plaats van nefrine, podocine en CD2AP van het celmembraan naar het cytoplasma. Deze relocatie kan opgeheven worden door serum van gezonde mensen. Het vinden van deze factor in het serum blijft een grote uitdaging voor vervolgstudies.

Behalve een erfelijke oorzaak, kan FSGS ook veroorzaakt worden door een immunologische afwijking, een virus of een toxine. Steroïden en cyclosporine zullen een steunpilaar blijven in de behandeling van FSGS. Deze behandeling is gebaseerd op het concept dat de laesies en proteinurie afkomstig zijn van een immunologische reactie. Bij PAN(puromycin aminonucleoside)-geïnduceerde podocyt schade zorgen steroïden voor bescherming en herstel door actine-filamenten te stabiliseren. Angiotensin II heeft een

dynamisch effect in de renale circulatie en veroorzaakt een verhoogde permeabiliteit van de nier voor albumine. Tevens veroorzaakt het een reorganisatie van het cytoskelet van muis-podocyten door relocatie van F-actine en ZO-1.
Zowel deze studies, als ook naar genetische afwijkingen zoals in dit proefschrift beschreven, laten zien dat onderzoek naar de biologie van de podocyt meer inzicht geeft in de pathogenese van het nefrotisch syndroom, en door beter inzicht wellicht kan leiden tot een meer gericht therapeutisch beleid.

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Curriculum Vitae

Marije Maria Löwik was born on March 4th, 1977 in Weerselo, the Netherlands. She attended high school in Oldenzaal, Thij College (VWO), and graduated in 1995. In the same year she started her HBO study in Enschede, HLO – medical biochemistry.
Her traineeship was performed at the Laboratory of the Department of Paediatrics, University Medical Centre St. Radboud, Nijmegen, under direct supervision of Drs. R.H. Triepels and Dr. L.P.W.J. van den Heuvel. The subject of traineeship involved mitochondrial disorders and more in detail genetic defects of complex I subunits.
After graduation in 1999, she started at this department as a research technician. She worked on the role of polymorphisms in the development of the Hemolytic Uremic Syndrome in close collaboration with Drs. D.M. te Loo and Ing. T.J. van der Velden under supervision of Dr. L.P.W.J. van den Heuvel.
In March 2000 she started as a PhD student and the results of her research project are described in this thesis.
From January 2005 till January 2008 she worked at the department of Molecular Neurobiology of the Biological Centre in Haren (University of Groningen) as a technician under supervision of Dr. U.L.M. Eisel.
In September 2007 she started the study HBO-V to become a nurse. For this reason she started to work in September 2009 as a student-nurse at the “Medical Spectrum Twente”, the hospital of Enschede. She hopes to finish this study within one year.
Marije lives together with Richard in Oldenzaal. They are the proud parents of Tycho and Iris.

List of publications

Löwik MM, Levtchenko EN, Monnens LA, van den Heuvel LP.
WT-1 and NPHS2 mutation analysis in patients with non-familial steroid-resistant focal-segmental glomerulosclerosis.
Clin Nephrol. 2003; 59: 143-146.

Löwik MM, Hol FA, Steenbergen EJ, Wetzels JF, van den Heuvel LP.
Mitochondrial tRNALeu(UUR) mutation in a patient with steroid-resistant nephrotic syndrome and focal segmental glomerulosclerosis.
Nephrol Dial Transplant. 2005; 20: 336-341.

Löwik MM, van den Berkmortel FW, Noordam C, van Hamersvelt HW, van den Heuvel LP, Levtchenko EN.
A genetic childhood disease with consequences in adult life: the Denys-Drash syndrome
Ned Tijdschr Geneeskd. 2005; 149: 1751-1755.

Löwik MM, Groenen PJ, Pronk I, Lilien MR, Goldschmeding R, Dijkman HB, Levtchenko EN, Monnens LA, van den Heuvel LP.
Focal segmental glomerulosclerosis in a patient homozygous for a CD2AP mutation.
Kidney Int. 2007; 72: 1198-1203.

Löwik MM, Levtchenko E, Westra D, Groenen P, Steenbergen E, Weening J, Lilien M, Monnens L, van den Heuvel L.
Bigenic heterozygosity and the development of steroid-resistant focal segmental glomerulosclerosis.
Nephrol Dial Transplant. 2008; 23: 3146-3151.

Löwik MM, Groenen PJ, Levtchenko EN, Monnens LA, van den Heuvel LP.
Molecular genetic analysis of podocyte genes in focal segmental glomerulosclerosis – a review.
Eur J Pediatr. 2009; 168 : 1291-1304.

Kyrieleis HAC, **Löwik MM**, Pronk I, Cruysberg JRM, Kremer JAM, Oyen WJG, van den Heuvel LP, Wetzels JFM, Levtchenko EN.
Long - term outcome of frequently relapsing minimal change nephrotic syndrome in children.
Clin J Am Soc Nephrol. 2009; 4: 1593-1600.